A Study of The Neuropathology of HIV Infection in an African Paediatric Cohort

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DECLARATION

I declare that the work of this thesis is my own composition and performance

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LIST OF ABBREVIATIONS

Abbreviation	Name
ADC	AIDS dementia complex
AE	adsorptive endocytosis
AIDS	acquired immuno-deficiency syndrome
APC	antigen presenting cell
APOE	apolipoprotein E
ARC	AIDS-related complex
βAPP	beta-amyloid precursor protein
BBB	blood brain barrier
CA	carbonic anhydrase
CD	cluster of differentiation
CDC	Centers for Disease Control and Prevention
CDK	cyclin dependent kinase
CMV	cytomegalovirus
CNP	2′, 3′- cyclic nucleotide 3′ -phosphodiesterase
CNS	central nervous system
Cpz	chimpanzee
CSF	cerebro-spinal fluid
CTL	cytotoxic T-lymphocyte
DNA	deoxyribonucleic acid
ECM	extra-cellular matrix
EBV	Epstein-Barr virus
gal	galactocerebroside
GFAP	glial fibrillary acidic protein
gp	glycoprotein
HAD	HIV-associated dementia
HAART	highly active anti-retroviral therapy
HHV	human herpes virus
HIV	human immuno-deficiency virus
HIVE	HIV-encephalitis
HIVL	human immunodeficiency virus leucoencephalopathy
HLA	human leucocyte-associated antigen
HSV	herpes simplex virus
HTLV	human T-lymphotrophic virus
ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
IRIS	immune reconstitution inflammatory syndrome
kD	kiloDalton
LAV	lymphadenopathy associated virus
LCA	leucocyte common antigen
LIP	lymphoid interstitial pneumonitis
LPS	lipopolysaccharide
LTR	long terminal repeat
MBP	myelin basic protein
MGC	multinucleate giant cell

MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinases
MMWR	Morbidity and Mortality Weekly Reports
MNGC	multi-nucleate giant cell
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
MTCT	mother to child transmission
NMDA	N-methyl-D-aspartate (as in receptor)
NO	nitric oxide
NRTI	nucleotide reverse transcriptase inhibitor
NNRTI	non-nucleotide reverse transcriptase inhibitor
MW	molecular weight
nm	nanometre
p as in p24	protein
p as p=0.05	probability
PCM	protein calorie malnutrition
PCNA	proliferating cell nuclear antigen
PCNSL	primary CNS lymphoma
PCP	pneumocystis pneumonia
PCR	polymerase chain reaction
PGL	persistent generalised lymphadenopathy
PLH	pulmonary lymphoid hyperplasia
PML	progressive multifocal leucoencephalopathy
PNS	peripheral nervous system
RB	retinoblastoma protein
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SIV	simian immunodeficiency virus
Tat	transcriptional activator protein
Th cell	T-helper cell
TB	tuberculosis
TGF	transforming growth factor
TLR	Toll-like receptor
TNF	tumour necrosis factor
VCAM	vascular cell adhesion molecule
WGA	wheat-germ agglutinin
WHO	World Health Organisation
ZO	zona occludens

Contents

Abstract

Background:

In 1991–92 a large collaborative post-mortem study of adults and children was undertaken in the West African city of Abidjan, Cote d'Ivoire, in order to document the impact of human immunodeficiency virus (HIV) infection on the already high mortality from infectious disease. As part of this original study, brain tissue preserved in paraffin wax blocks was retained from 78 HIV positive children (76 HIV-1 and 2 HIV-2) and 77 age-matched HIV-negative children. The baseline neuropathological findings were published and the material was stored as an archive at the Western General Hospital, Edinburgh. Further study of this unique collection of cases was clearly warranted.

Hypotheses and Aims of the Present Study:

In the **Initial Study,** an attempt was made to quantify white matter cell numbers using a small sample of the whole cohort. The hypothesis was that it would be possible to obtain reproducible numbers for total cell counts in the white matter of HIV negative children and that the numbers were likely to be significantly higher in HIV positive children. The **Main Study** focussed on the extent of inflammatory change in HIV negative and positive cases, with the prediction that HIV positive children would show a higher level of inflammatory infiltrate than HIV negative children, despite the high level of

background brain pathology in the latter group, and that this would be associated with evidence of greater white matter damage in the HIV positive cases. Lastly in the **Apolipoprotein E (APOE) Study**, it was predicted that the inflammatory response, particularly the degree of microglial activation would be influenced by the APOE genotype, being most evident in children possessing one or more APOE ε4 alleles.

Materials and methods:

For the **Initial Study**, eight age-matched HIV negative and positive cases were selected from the African cohort, together with an additional HIV negative case at either end of the age spectrum. Four regions of the brain were selected from each of these 10 cases, including cerebral convexity, hippocampus, basal ganglia and the cerebellum. Sections were stained routinely with haematoxylin and eosin, Luxol fast blue and by immunohistochemistry. Total cell counts were performed in an area of one mm^2, and subsequently for individual cell types identified using a combination of simple morphology and immunostaining for glial fibrillary acidic protein (GFAP). Although there is no immunostain that reliably identifies all of the members of a particular glial population, whether they be astrocytes, oligodendrocytes or microglia, separation of these cell types was attempted on the basis of GFAP positivity or on simple nuclear morphology. The results were tabulated in Excel.

In the **Main Study** 40 further cases from the African cohort were examined. These included 20 HIV negative and 20 positive, chosen to represent three age

groups, lower, middle and upper, up to the age of six years. The basal ganglia and the hippocampus were selected for examination in these cases. In about half of these cases, significant pathology other than HIV-associated changes was present and the spectrum of CNS disease included malaria, meningitis and non-HIV encephalitis. Sections were stained routinely with haematoxylin and eosin, Luxol fast blue and by immunohistochemistry for inflammatory cell markers (CD14, CD16 and CD68) or lymphocyte markers (CD8 and CD20). Sections were examined by routine microscopy, and by simple quantitation or image analysis.

In the **APOE study** genotyping was undertaken first on the forty cases used in the main study. Twenty further cases were added subsequently, 10 HIV positive and 10 negative, and within the same age range as those in the main study. DNA was extracted from paraffin sections according to a standard protocol and amplified using polymerase chain reaction (PCR). The degree of inflammation, as detected by CD16 and CD68 image analysis in these cases, was correlated with the different APOE genotypes.

All cases used in this study were anonymised. Ethical permission was sought and obtained for this study **(LREC/2003/6/6).**

Results:

The results of the **Initial Study** showed that the total cell numbers varied from case to case in the HIV negative group, and from one brain region to

another, but that these differences were not statistically significant. The total cell counts also varied between individual HIV positive cases and these differences were not statistically significant either. However, comparison of the means for HIV negative cases with those of the HIV positive cases revealed significant increases in the latter group. Despite the obvious limitations of the morphological approach to individual cell typing, subset counts from the cerebral convexity, basal ganglia, hippocampus and cerebellum, putatively of astrocytes, oligodendrocytes, microglia and endothelial cells, showed significantly higher levels in the HIV positive cases.

In the **Main Study**, changes in the white matter assessed by routine and Luxol fast blue staining, and by immunohistochemistry for myelin basic protein (MBP), β-amyloid precursor protein (BAPP) and GFAP, and graded according to a simple system, showed only limited differences between HIV positive and negative groups. A general increase in activation of macrophage/microglial cells and of lymphocyte numbers was found in both basal ganglia and hippocampus in the HIV positive group. This increase in the HIV positive group was detected despite a reasonable balance of background brain pathology between the HIV positive and negative subsets. In the case of CD68 immunostaining, the increase was statistically significant for the grey ($p=0.002$) and white ($p=0.009$) matter of the basal ganglia. For counts of CD8 positive perivascular cells, the increase was also significant in grey ($p=0.001$) and white matter ($p=0.000$) of the basal ganglia, and in grey ($p=0.002$) and white ($p=0.000$) matter of the hippocampus.

In the **APOE study**, DNA extraction was successful in 47 cases in total. The

distribution of APOE ε3 and ε4 alleles in this group proved in accordance with the known West African distributions where the ε4 allele approaches a frequency of up to 29%. In this study, though the case numbers are small, the frequency of the ε4 allele was 41% in HIV negative children and 23% in HIV positive children (p=0.01). These rates were significantly higher than in most Caucasian populations (p=0.001). Satisfactory immunostaining for inflammatory markers was achieved in 44 of the genotyped cases. Correlations with the degree of neuroinflammation in grey and white matter of the basal ganglia were confined to APOE ε3/ε3 and APOE ε3/ε4 cases because of the small numbers of cases with rarer APOE allelic patterns. For HIV negative children, there was little difference for CD16 or CD68 staining between APOE ε3/ε3 and APOE ε3/ε4 cases, either in white or grey matter. For HIV positive children, subjects with ε3/ε4 genotypes did show a trend for higher levels of CD68 activation than those with 3/3 genotypes, particularly in the basal ganglia white matter, but failed to reach significance (p=0.07 for white matter and p=0.1 for grey matter).

Conclusions:

1. Although the results of the initial study are limited in their possible application to any studies other than in this cohort, the increases in total and individual cell counts in the HIV positive group are intriguing. The limitations of a study based on morphological assessment are recognised.

2. In the main study the failure to demonstrate significant white matter change in either HIV negative or positive groups may mean that there is none, but wider sampling in each case and examination of a larger number of cases would increase confidence in this result. It is noted that incomplete myelination in these young subjects is a possible confounding factor in this study. Inflammatory changes, both innate and acquired, proved to be significantly greater in both grey and white matter of HIV positive compared with negative cases, there being a relative balance of co-existing disease in the two groups. This study shows, as predicted, that HIV infection is accompanied by significant microglial activation in these West African children, and that this occurs even in the absence of productive viral infection in the brain. This finding is in keeping with what is known in Western paediatric populations infected with HIV.

3. APOE genotyping revealed a distinctive allele distribution especially in respect of the ε4 allele, which was found to be twice as common in this African cohort as in the Scottish population. Although there was trend for greater microglial activation in APOE ε4 HIV positive children, the differences between these and APOE ε3 HIV positive children did not reach significance.

Prologue

In Rome, in the first century A.D., Pliny the elder wrote: "Semper aliquid novum ex Africa" (there is always something new from Africa). The reference was to the Greek observation concerning unusual forms of plant or animal life. The latter included animals of ferocity or size such that, in gladiatorial combat in the arenas, a public thirst for spectacle could be assuaged. It was not known then, of course, that mankind itself had originated in, and spread from, Africa and it could not have been foreseen, even by the Sybil, the ancient prophetess, that something much smaller than a carnivore would emerge from Africa to prove a more formidable foe; and take as its arena the whole world, rendering the word 'decimation' redundant by several orders of magnitude. The most recent estimates of numbers are 33 million infected with HIV/AIDS in 2007, 2.5 million became newly infected and 2.1 million deaths for the same year (WHO 2007).

Chapter 1: General Introduction

1.1 - HIV/AIDS – The Beginning:

Human immunodeficiency virus (HIV) infection was recognized for the first time in 1981 in the USA when an epidemic of illness occurred in homosexual men in New York, Los Angeles and San Francisco. They were found to be suffering from immunodeficiency associated Pneumocystis pneumonia. Within a year, further cases, with Central Nervous System (CNS) involvement, were reported (Britton and Miller 1984). Though these, at post mortem, showed encephalitis with Cytomegalovirus (CMV) or toxoplasma, an additional pathological process was suspected.

Epidemiological studies of further populations of similarly afflicted individuals suggested that a transmissible agent was responsible (Francis et al. 1983) and this was identified as a retrovirus (Barre-Sinoussi 1983). It was shown to have a strong relationship on molecular hybridisation analysis (Gonda et al. 1985) to another retrovirus, Visna virus, the causative agent of a chronic nervous system disease of sheep (Sigurdsson et al. 1957).

Common features of this new human infection, such as oral candidiasis, recurrent Herpes infections, *Pneumocystis carinii* pneumonia and Kaposi's sarcoma suggested a defect in cellular immunity. Careful study revealed that the natural history of the disease was one usually with a time course of some years terminating with a variety of illnesses, all having as their basis severe progressive impairment of immunity. The condition became known as the

Acquired Immunodeficiency Syndrome (AIDS) and the associated terminal conditions were referred to as AIDS defining illnesses.

Other sections of the community affected in smaller numbers were haemophiliacs, intra-venous drug users and those with a history of recent blood transfusion. These characteristics suggested a blood-borne and sexually transmissible agent and, two years later, in France this was identified as a retrovirus, and provisionally named lymphadenopathy associated virus (LAV)(Barre-Sinoussi et al. 1983) or Human T-lymphotrophic virus III (HTLV III). In 1986 the International Committee for the Taxonomy of Viruses suggested the name Human Immunodeficiency Virus (HIV) that was adopted, and is still retained as HIV -1.

Though initially it appeared that this was confined to America, and then Europe as well, evidence accrued to demonstrate that at the outset it was already present in four continents. Heterosexual transmission appeared to occur as readily as homosexual, as was apparent in Africa. A second related retrovirus was found in West Africa, and subsequently called HIV-2. It has remained largely located there, whilst HIV-1 has a worldwide distribution and is responsible for the pandemic now affecting some 33 million people.

1.2 - The Virus

Sub-Saharan Africa is now known to be the place of origin of the Human Immunodeficiency Virus in its two distinct types HIV-1 and HIV-2. HIV is a member of the family of viruses known as the retroviruses, which infect

principally vertebrates, causing a spectrum of disease including malignancies, immune deficiencies and neurological disorders. They share a common structure, genome and mode of replication in which viral RNA is transcribed to DNA, which is then incorporated into the host genome. HIV is further classified into a sub-family known as the lentiviruses, which cause slowly developing illnesses in animals - such as Equine Infectious Anaemia, Feline Immunodeficiency Disease and Visna, a demyelinating disease of sheep. The lentivirus diseases of primates, including Simian Immunodeficiency Diseases (SIVs) and HIV-1 and HIV-2 show tropism for a cell-surface receptor on lymphocytes designated Cluster of Differentiation 4 (CD4). Hallmarks of their behaviour, apart from insidious disease onset and persistence, are latency and a remarkable capacity for genetic variation. Disease transmission is both horizontal and vertical.

The principal reservoir of African lentiviruses is in the family of Old World Monkeys and most are species-specific (Hahn et al. 2000). Though termed Simian Immunodeficiency Viruses (SIVs), because of their close similarity to HIV-1, they do not cause disease in their natural hosts. When, in 1989, an HIV-1 related lentivirus was detected in wild chimpanzees (Peeters et al. 1989), (Huet et al. 1990), the suspicion was that this could be the zoonotic source of HIV. This virus, designated SIVchimpazee (cpz), appeared closer antigenically to HIV-1 than to HIV-2 or the other SIVs. Subsequently this virus was found rarely, and only in captive chimpanzees (Gao et al. 1999), (Nerrienet et al. 2005). More recently extensive field studies in Cameroon, using faecal specimens gathered from the forest floor and analysed for SIVcpz

nucleic acids, have suggested that for the chimpanzee subspecies, *Pan troglodytes troglodytes*, community prevalence rates of infection are of 29-35% (Keele et al. 2006).

Genetic analysis of HIV-1 and HIV-2 has allowed further classification of HIV-1 into 3 main groups, M, N and O, of which type M is responsible for the present pandemic; and HIV-2 into 6 subtypes, A-F (Human retroviruses and Aids – Los Alamos 1998) (Hahn et al. 2000). HIV-1 group M is considered to have evolved directly from SIVcpz, which itself is thought to have arisen as a recombinant of ancestral forms of SIVs, currently infecting red-capped mangabeys and *Cercopithecus* monkeys in West central Africa (Bailes et al. 2003). HIV-2 is thought to have arisen from the sooty mangabey monkey.

HIV-1 sequence analysis suggests the date of the last common ancestor of the main group of HIV-1 to be 1931 (Korber et al. 2000). The species jump - chimpanzee to man - is thought to have occurred in or near Cameroon, perhaps as a result of blood exposure in the bush meat trade, and to have had as its first staging post a large town, such as Kinshasa (Vidal et al. 2000).

A natural concern, frequently expressed, is that any of the many other SIVs found in African monkeys today present a continuing zoonotic risk (Hahn et al. 2000), (Keele et al. 2006).

Fig. 1.2.1 - The Organisation of the HIV Genome and Protein Derivatives

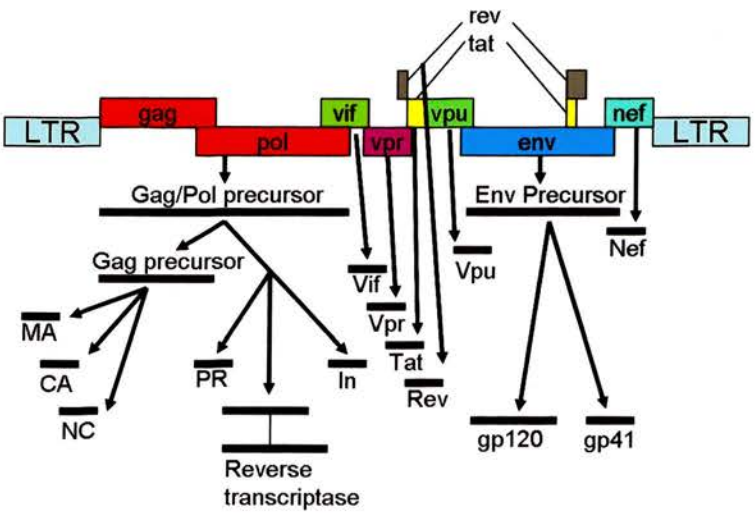


Fig. 1.2.2 - The Replication Cycle of HIV

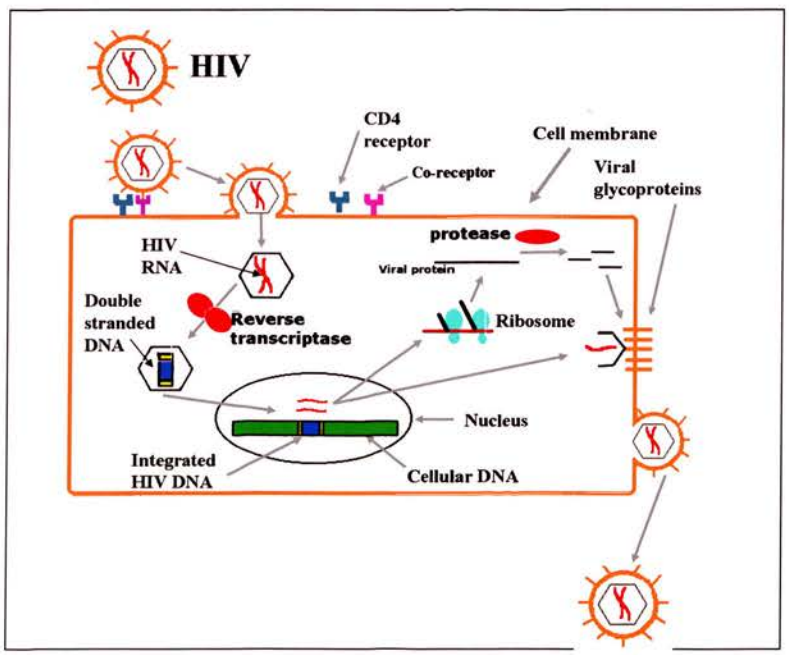


Table 1.2.1 - The structural HIV genes and their proteins

Gene	Protein	Location/Function
Gag	p17	Matrix Protein
	p24	Capsid Protein
	p9	Nucleocapsid protein
Pol	p10	Protease
	p66/p51	Reverse transcriptase
	p32	Integrase
Env	gp41	Transmembrane envelope glycoprotein
	gp120	External envelope glycoprotein
Vpr	p15	Virus particle component

Table 1.2.2 - The regulatory HIV genes and their proteins

Gene	Protein	Function
Tat	p14	Transcriptional and post- transcriptional regulator of gene expression
Rev	p19	Post-transcriptional regulator
Nef	p27	reduces cell surface expression of the CD 4 receptor
Vif	p23	Virus infectivity factor essential for replication in macrophages and primary T cells
Vpu	p15	Membrane protein facilitating viral maturation and release

HIV is an enveloped retrovirus and the virion contains two copies of the RNA genome (see Fig. 1.2.1) The inner core proteins, coded for by the gag gene, are p9 – the nucleocapsid protein, p17 – the matrix protein and p24 – the capsid protein (see Table 1.2.1). The diagnosis of HIV infection is based on the finding of serum antibodies directed against gp41. The pol gene codes for three key enzymes, protease, reverse transcriptase and integrase that are involved in the replication cycle. The regulatory genes are shown in Table 1.2.2.

After entry into the body the process of infection begins when the envelope protein, gp120, binds to the main receptor CD4 on the surface of T4 lymphocytes. This results in a change in the gp120 molecule such that gp41 becomes accessible to bind to the co-receptor CXCR4 and fusion of the viral envelope with the cell membrane occurs. The virion then enters the cell and the viral RNA is copied into DNA by the enzyme, reverse transcriptase.

The viral genome is then incorporated into the host cell genome through the agency of the enzyme, integrase. The next step is the transcription of viral genes into viral messenger RNA (mRNA) by host cell polymerase. Viral assembly follows in the cytoplasm and the capsid proteins are elaborated by the enzyme, protease. The virion then leaves the cell by budding, acquiring the envelope from the cell membrane as it departs (see Fig.1.2.2).

1.3 - The Stages of HIV/AIDS

Several systems of classification have been used to describe the stages in the natural history of HIV/AIDS. The Centers for Disease Control (CDC) proposed a scheme based on clinical features with 4 principal groups:

1. Acute infection
2. Asymptomatic infection
3. Persistent generalised lymphadenopathy
4. Other disease

Group 4 is further divided into subgroups 4A-4E depending on the nature of the illness and these are:

Group IV A	HIV wasting syndrome (AIDS)
Group IV B	HIV encephalopathy – AIDS/HIVE and neurological disease
Group IV C 1	Major opportunistic infections specified as AIDS defining
Group IV C 2	Minor opportunistic infections
Group IV D	Cancers specified as AIDS-defining e.g. primary CNS lymphoma
Group IV E	Other conditions e.g cytomegalovirus pneumonia

In 1989 the World Health Organisation (WHO) offered a classification of HIV infection in adults based on clinical criteria and lymphocyte counts, the

groups ranging from asymptomatic individuals with CD4+ T-cell counts > 500 cells / cu. mm., to those terminally ill with counts < 200 cells /cu.mm. Now a finer grading correlates diseases with counts < 200 cells/cu.mm, e.g. *Pneumocystis carinii,* <100 cells/cu.mm, cerebral toxoplasmosis and < 50 cells/cu.mm, disseminated *Mycobacterium avium intracellulare.*

1.4 - Viral Entry into the Body

Several routes are involved: in adults via the genital or rectal mucosa in sexual intercourse, or by transfusion of infected blood or blood products, or through intra-venous drug misuse. In the foetus it can occur by trans-placental spread and at parturition; and in the infant, via the intestinal mucosa from maternally infected milk.

The efficiency of genital or rectal mucosal transmission is determined by a number of factors, not least being the HIV load in semen. Usually these mirror blood levels and are lower, though may be higher, especially in the setting of co-existent infection such as CMV (Sheth et al. 2006). Also the time with respect to seroconversion is important, especially high levels in semen occurring within the first two months after infection (Pilcher et al. 2004). Studies using human cervico-vaginal organ culture systems have shown early binding of virions to cervical mucosa with penetration to sub-mucosa within 3-4 days (Maher et al. 2005). Cells infected had the morphology and distribution

of macrophages, and yet even after 7 days CD 4+/CD3+ cells showed no evidence of infection (Palacio et al. 1994).

However, several modes of viral uptake are possible and differing views are expressed as to the main route. When cell-free virus is considered, infection of the target cell is determined by its expression of co-receptor CD4 and chemokine receptor CCR5 (Doms 2001). SIV infection in rhesus monkeys has been used as a model to study the dynamics of initial infection, and these have suggested, contrary to the above, that the earliest and most marked infection occurs within CD4+ T cells of the mucosa (Stahl-Hennig et al. 1999) rather than Langerhans dendritic cells (Veazey and Lackner 1998; Zhang et al. 1999; Vajdy et al. 2000; Veazey et al. 2000). These latter cells not only are less permissive for viral replication, but may actually inhibit viral replication through the agency of the C-type lectin langerin (CD 207) expressed in the Birbeck granules of Langerhans cells. Experimental blocking of langerin using a monoclonal antibody rendered Langerhans cells more liable to HIV infection and more efficient in transmission to CD4+ lymphocytes (de Witte et al. 2007).

Cell-to-cell transmission by viruses is considered much more efficient than cell-free spread (Dimitrov et al. 1993) in terms of time – even within minutes (Sato et al. 1992) – and survival, by relative shielding from the immune response. For human T cell leukaemia virus type 1 (HTLV-1) it is the principal pathway (Igakura et al. 2003) as infected lymphocytes release very few HTLV-1 viral particles. The extent to which this mode of spread operates in HIV infection is unknown but it occurs readily (Sherer et al. 2007).

1.5 - The Immune System and Acute HIV Infection

The tissues of the body that bear the major brunt of HIV infection are the immune system and the CNS. In the primary illness the clinical expression is principally due to immune system involvement. Though the immune system is covered in detail in a later section, a short introductory account of the immunodynamics of early HIV infection is given here.

In the first 2-3 weeks following transmission, by whatever route, the virus becomes established in the lymphoid tissues of the host (Haase et al. 1996). Seroconversion, marked by the appearance of anti-HIV antibodies, to core and envelope proteins, occurs within 3-8 weeks of infection. The chief cell infected by HIV is the CD4+ lymphocyte, and to a lesser extent the CD4+ macrophage. As explained later in the introduction most circulating lymphocytes, 60-70%, are naïve or unprimed and a similar proportion is found in the T-cell zones of lymph nodes and the spleen. These mature T lymphocytes bear the CD 3 (cluster of differentiation) molecular complex, known as the T-cell receptor, on their surfaces. In activation further molecular complexes are expressed to give two main lymphocyte subsets, CD4+ and CD8+ T cells.

The activities of the two types are quite distinct though not necessarily entirely so. The majority of CD8 cells perform, in their activated state, a cytotoxic function, especially in viral infections, where infected host cells bearing viral products on their surfaces are identified and killed. The CD4 lymphocytes, also known as T helper or Th cells, produce, when activated,

signalling molecules known as cytokines which influence the numbers and activities of B-cells, cytotoxic T cells, and macrophages.

The HIV envelope glycoprotein, gp120 , has a particular affinity for the CD4 molecule (also expressed by monocyte/ macrophages and dendritic cells), but productive infection in the CD4+ cell was found to depend on the presence of other receptors, known as co-receptors CCR5 and CXCR4 (Berger et al. 1999). These chemokine receptors are used differentially by specific HIV strains, predominantly CCR5 for macrophages and CXCR4 for T cells.

The details of viral binding and entry are complex but essentially involve firstly the conjunction of gp120 envelope glycoprotein and the CD4 molecule, then the linkage of gp120 to either of the co-receptors and finally, through the agency of gp41, cell membrane fusion and viral entry (Littman 1998).

The sequelae depend on the state of activation of the invaded CD4+ T cell. In inactive cells the virus may remain in pro-viral form in the cytoplasm, but in dividing cells the virus enters the nucleus and becomes part of the genome. Latency or productive infection, with cytolysis, may follow (McCune 2001).

The natural ligand for the CD4 molecule is the major histocompatability complex (MHC) class II molecule, but its binding affinity is exceeded by that of the HIV external envelope protein, gp120.(The MHC is a complex of genetic loci that encodes a family of cellular antigens. There two principal classes that code for leucocyte antigens – human leucocyte antigens (HLA) classes I and II. MHC class I peptides are expressed on the surfaces of all nucleated cells and allow cytolytic T-cells to recognize cells infected with pathogens. MHC class II molecules are expressed on B cells, dendritic cells

and macrophages, that function as antigen presenting cells (APCs) for the activation of CD4 helper T cells.) The effect of infection in the case of lymphocytes is usually a cytopathic one, and eventually the replacement capacity of the bone marrow is overcome, total CD4 lymphocyte levels fall and a state of immunodeficiency supervenes. Some infected lymphocytes do not succumb, and remain the site of active infection. Despite infection, the numbers of monocytes and macrophages remain unchanged. The numbers infected are relatively less than CD4 lymphocytes and the cytopathic effect much less pronounced (Schnittman et al. 1989).

By the time seroconversion occurs, viral replication is both rapid and on a very large scale - it is calculated that daily production of virions by infected CD4+ T cells is in the order of several billions (Zhang et al. 1999), and some 10-100 million infected CD4+ T cells die daily (Wei et al. 1995). Resting infected CD4+ T cells may number 1-100 million (Embretson et al. 1993), and there is also a large reservoir of virion-associated follicular dendritic cells (Fox and Cottler-Fox 1992). The profound state of immunodeficiency that later develops has as a cornerstone the depletion of the CD4+ T lymphocyte population as a direct result of infection, but related factors operate. Chronic activation of uninfected cells may lead to their depletion by apoptosis (Hazenberg et al. 2000). Also decreased production, by loss or interference with maturation of precursor cells in the thymus (Wolthers et al. 1996), is probably just as important.

The thymus is a lymphoid organ, situated in the upper mediastinum, that reaches its maximum size in early adolescence with a weight of 30-40 gm.

After that it gradually involutes, but maintains a degree of function into old age. In the embryo, at about 8 weeks of age, it is invaded by immature lymphocytes, the first from the yolk sac and the liver in their haemopoietic phases, and then from the bone marrow. The major role of the thymus is in the functional differentiation and selection or deletion of T-lymphocytes. The immature lymphocytes in the cortex do not possess the T-cell receptors on their surfaces - they are CD4 and CD8 negative. The maturation process is a complex one and involves the agency of specialized cells, called thymic nurse cells and thymic hormones. Lymphocytes reactive to self-antigens are eliminated by apoptosis, and the mature CD4 and CD8 lymphocytes are released into the general circulation.

Maldevelopment of the thymus, as in the Di George syndrome, is characterised in childhood by a state of impaired immunity commonly associated with death from infection (the role of the thymus in childhood HIV infection is considered on page 82).

From the clinical point of view, in some 70% of cases there is a transient glandular fever type illness within the first two to three weeks of infection and, in a few, a neurological syndrome with a mild meningitis or encephalitis. In the plasma the CD4 lymphocyte count falls from its normal level of about 1,000 per μl to 300-400 cells per μl and the plasma HIV-RNA level may rise to1 million copies/ml. The antibody to the envelope protein, gp41, persists indefinitely and its detection is the basis for identification of HIV infection.

By about 12 weeks the CD4 lymphocyte count has risen to near its original value and the HIV-RNA level has fallen to a low stable level. These values,

reflecting a balance between the host's immunity and the virulence of the infecting HIV strain, were found to be an accurate predictor of the time course in any particular individual until the onset of AIDS, and the onset of this steady state has become known as the viral set point (Mellors et al. 1997). Recovery from the illness of primary infection is usually complete and the illness itself may be recalled only in retrospect.

1.6 - Asymptomatic Phase of the Infection

After the primary illness subsides, a long asymptomatic period ensues. The median period from infection to the next stage is 5 -10 years.

Throughout the asymptomatic period persistent generalised lymphadenopathy (PGL), defined as enlarged lymph nodes at two or more extra-inguinal sites, is a variable feature. Plasma viral load in untreated individuals remains elevated, and the fall in CD4+ T-cell levels may be in the order of 150 cells/μl of blood per year.

During this latent stage of HIV infection, a number of factors operate that may modulate the severity and rate of progression of the disease. On the one hand, there is a small sub-set of individuals, termed "non-progressors" or "elite controllers", who remain healthy despite being infected for many years. AIDS associated alleles, (they are alternative forms of a gene pair that may be associated with either an enhanced or suppressed rate of progression of HIV infection) are described below. The key features of this group, in which the host adaptive immune response is important through HIV-specific CD4+ and

CD8+ T-cell actions (Pantaleo et al. 1995; Betts et al. 1999), are a low level viraemia and a stable CD4+ T-cell population. Host and viral genetic factors underlie the equilibrium – the prolonged survival of 28 - 40% of a group of Caucasian HIV subjects to 10 or more years was associated with their being fully heterozygous at HLA class 1 loci or their lack of AIDS-associated alleles (Carrington et al. 1999).

The HLA class 1 system is of importance in viral infections for two reasons. HLA class 1 has been shown to bind to viral peptides, thereby influencing the activation of cytotoxic T lymphocytes and their action on the virus. Also class 1 molecules similarly interact with natural killer cells through their surface receptors, resulting the elimination of virally infected cells .These two cell sets therefore play a crucial role in the establishment or otherwise of early and continuing viral infection.. Two of the HLA alleles that have been shown to influence the course of HIV infection are, one, HLA-B*27, that is associated with slow progression and the other, HLA-B*35 with a more rapid course (Carrington and O'Brien 2003) A long quiescent period may be due to deletions or mutations in the HIV nef gene. 'Controllers' showed higher numbers of IL-2 producing CD4 + lymphocytes than do non-controllers (Emu et al. 2005).

Age is a factor which influences the rate of progression - beyond an age of about 50 years the risk of progression increases by 40% for each decade of life at the time of infection (Darby et al. 1996). The older the subject at infection, the more likely a rapid course (Belanger et al. 1997).

Eventually, in most members of this heterogeneous group, viral replication increases and CD4+ T-cell numbers fall, due to several mechanisms including abnormal signalling of CD8+ T cells, decreased stores of perforin, increased HIV mutant escapes and abnormal B and T cell differentiation (Koenig et al. 1995; Goulder et al. 1997; Appay et al. 2000).

There also exists a very small group of people who remain uninfected despite repeated exposure to HIV-1 through sexual contact, and this capability has been shown to be due to their being homozygous for a deletion in the CCR-5 gene which encodes an essential co-receptor, now known as CCR-5 (Liu et al. 1996). The resultant defective protein is shortened, and is undetectable at the cell membrane level.

The fact that productive infection of HIV occurs much more readily in activated T-cells (McDougal et al. 1985; Zagury et al. 1986) suggested that coexisting microbial disease might enhance progression. Support for this view was obtained in serological studies involving HIV positive American homosexual men and African sufferers of both sexes. Evidence of previous exposure to diverse viruses such as HSV, CMV, EBV and Hepatitis B (all known to perturb the immune system) was found in both groups compared with controls (Quinn et al. 1987). Mycobacteria infections provoke the secretion of large amounts of cytokines, including tumour necrosis factor alpha (TNF-α), interleukin I beta (IL-1β) and interleukin 6 (IL-6), which all stimulate the replication of HIV in monocytes and macrophages (Poli et al. 1994). *Mycobacterium avium* infections commonly give rise to bacteraemia in

AIDS sufferers in the USA, and in Africa *M tuberculosis* is one of the commonest terminal opportunistic infections (Lucas et al. 1993).

The situation regarding African populations is of particular concern by virtue of the enormity of the epidemic, where infection rates of 30% or more were returned from several African countries (Merson 1993), with devastating effects on infrastructure and economy. The background level of diverse infection is higher in African populations compared to affluent communities and the degree of associated immune activation, even in the HIV negative population, is a notable feature (Bentwich et al. 1995). One very significant contributor to this infective burden, and which is a chronic one, is helminth infestation (Nahmias et al. 1993). Protozoan infection with *Toxoplasma gondii*, *Leishmania donovani* and *Plasmodium falciparum* (Kublin et al. 2005) are others.

The next stage of mildly symptomatic disease (CDC classification category 4B) is characterised by the appearance of conditions suggestive of mild impairment of cellular immunity, and correlating with CD4 T-cell counts of below 500 / cu.mm but greater than 200 /cu.mm. These conditions may be general, such as weight loss, diarrhoea, fever and night sweats or specific, such as oral hairy leucoplakia, cervical dysplasia and herpes zoster. Fungal and bacterial skin conditions are troublesome and common.

1.7 – AIDS

The terminal stage of HIV infection (AIDS) is defined by the development of conditions associated with severe immunodeficiency – CD4 cell counts of less than 200 cells /cu.mm. There are a number of such specified conditions and they include both common and unusual (opportunistic) infections, several types of neoplasm including lymphoma, and several illnesses of the central nervous system, for example:

Table 1.7.1 - Some major AIDS-defining conditions

Nature	Condition	Location
General		
	Wasting Syndrome	General
Infections		
	Candidiasis	Oesophageal
	Coccidiomycosis	Disseminated
	Cryptococcosis	Disseminated
	Cytomegalovirus	Retinitis
	Pneumocystis Carinii	Pulmonary
	Mycobacterial Disease	Disseminated
	Mycobacteria Tuberculosis	Pulmonary
Neoplasms		
	Invasive Cervical Cancer	Cervix
	Kaposi's Sarcoma	Disseminated
	Non-Hodgkin's Lymphoma	Lymphoid
CNS specific		
	Cerebral Toxoplasmosis	CNS
	Primary Cerebral Lymphoma	CNS
	HIV Encephalopathy	CNS

The more important of these are now considered in relationship to the systems they commonly affect.

Respiratory System

The first cases seen in 1981 suffered from an unusual pulmonary condition, ***Pneumocystis carinii pneumonia,*** which is caused by an organism with fungal and protozoal features. Patients present with shortness of breath of several weeks' duration, malaise, fatigue and a dry cough, and x-ray may show some peri-hilar shadowing. The differential diagnosis includes pneumonia caused by bacteria, including tuberculosis (TB). The diagnosis is confirmed by sputum cytology and treatment with co-trimoxazole is usually effective. *Pneumocystis* pneumonia is still the commonest AIDS-defining illness, though triple anti-HIV therapy and primary prophylaxis have reduced the incidence.

Pulmonary infection with *Mycobacterium tuberculosis*, causing TB, is a major problem in the pandemic, as about a third of the 33 million HIV sufferers are also infected with TB and most of these live in sub-Saharan Africa. Access to treatment is limited and compliance often suboptimal. Multi-drug resistance is common and the two diseases have an adverse effect on each other. Bacterial pneumonias, due to *S. pneumoniae* and *H. influenzae*, are more common in HIV infection and more severe.

The Gastro-Intestinal Tract

Several AIDS-defining conditions affect the gastro-intestinal tract. Oesophageal candidiasis can be severe and cause dysphagia. Diagnosis is

confirmed by barium swallow or endoscopy. Anti-fungal agents are an essential part of treatment. Two groups of zoonotic protozoan parasites, *Cryptosporidium* and *Microsporidium,* commonly cause severe diarrhoea and malabsorption. Hydration and treatment with albendazole are important, but success is limited unless highly active antiretroviral therapy (HAART) is used to restore a low CD4 count.

Cytomegalovirus is implicated in gastro-intestinal tract disease if the CD4 count falls below 100 cells / cu.mm. It can cause oesophagitis and colitis and without HAART the prognosis is poor. Another disease due to a Mycobacterium, *M. avium intracellulare,* which may originate in either the gastro-intestinal or respiratory tracts, has a tendency to become disseminated but the incidence has fallen appreciably with HAART.

The Nervous System

Apart from infection with HIV itself, both in the primary infection and in end stage disease, the brain, spinal cord and peripheral nervous system (PNS) are subject to several opportunistic infections and other conditions that are AIDS related.

a. Meningitis or meningoencephalitis due to infection with the yeast *Cryptococcus neoformans* is an **AIDS**-defining illness of subacute onset with headache, fever and mild confusion. It occurs in about 5% of AIDS sufferers. Raised intracranial pressure with papilloedema may be present. The organism can be seen on direct film of the cerebro-spinal fluid (CSF) and obtained by culture. Anti-fungal therapy may not prevent complications, such as deafness

and blindness, and drainage procedures are indicated for raised intracranial pressure. The death rate is between 5 and 10%.

b. Cerebral toxoplasmosis: the protozoan parasite, *Toxoplasma gondii,* has a worldwide distribution. The natural host is the cat and transmission is by the excretion of cysts that are subsequently eaten by other animals. On ingestion tachyzoites are released from the oocyst and multiply intracellularly with the formation of cysts, especially in skeletal muscle and brain, and which remain viable indefinitely. Infection in the immunocompetent human is not uncommon – seropositivity levels in adults can vary between 20-70% - and results in a minor glandular fever-like illness. Transmission from an acutely infected pregnant woman to her foetus can result in serious congenital disease. In AIDS, waning immunity allows activation of any cerebral cysts, resulting in local or diffuse disease with necrosis and abscess formation. The clinical presentation is often an encephalitic one, and diagnosis may be difficult as the scan appearances of cerebral toxoplasmosis and lymphoma may be indistinguishable. A therapeutic trial of pyrimethamine may be conclusive but, if not, biopsy is indicated. Despite HAART the outcome is often poor.

c. Cytomegalovirus (CMV): a member of the herpes group of viruses, is of worldwide distribution and infects man and other mammals. In immunocompetent hosts the illness of infection is trivial and short lived. The virus can be found in most tissues and derives its name from its ability to cause the cells in which it resides to become larger, and to show both nuclear and cytoplasmic inclusions. It shares with other herpes viruses the habit of latency, and of becoming reactivated under certain conditions; it can be found in all

body fluids. Seropositivity in HIV positive groups varies between 50 and 75%, and before the advent of HAART, CMV retinitis was common in patients with CD4 cell counts below100 /cu.mm. Now a 90% reduction in incidence has been recorded in patients on long-term HAART. CMV also causes encephalitis and myelitis. Treatment with anti-viral agents such as ganciclovir is imperative to reduce the risk of blindness.

d. Progressive multifocal leukoencephalopathy (PML**)** is a condition first described in 1961 (Richardson 1961). This disease, characterised by progressive multifocal demyelination, was suspected of having a viral aetiology on account of inclusion bodies seen in pathological oligodendrocytes. It tended to occur in people in the older age groups associated with lymphoproliferative disorders or other neoplasms. In 1969 electron micrographs showed an array of viral particles in the nuclei of infected oligodendrocytes and these appeared similar to polyoma virions (Zu Rhein 1969).

In 1971 the virus was isolated from the brain of a patient with Hodgkins disease who had developed PML (Padgett et al. 1971), and it was named JC virus (JCV) after the initials of the patient. It was classified as a polyomavirus. This virus is widespread in nature, with sero-conversion occurring in childhood and some adult populations having a sero-prevalence rate as high as 90%. PML remained a rare disease, though numbers increased with the advent of organ transplantation and drug-induced immuno-suppression. Now, in AIDS, the incidence can be as high as 5-10% and, pre-HAART, it was the AIDS defining illness in 1-3% of cases. The prognosis is poor, most patients

surviving only a matter of months after diagnosis (by MRI scan) and despite HAART and/or anti-viral therapy.

e. Primary CNS lymphoma: in immuno-suppressed individuals the commonest CNS neoplasm is Primary CNS lymphoma (PCNSL) and it occurs in approximately 5% of all AIDS cases. The presentation is a short illness with headache, fever and increasing drowsiness – focal signs and seizures develop, and in an HIV/AIDS setting the principal differential diagnosis is cerebral toxoplasmosis. Failure to respond to anti-protozoal therapy, especially if toxoplasma serology is negative, suggests the diagnosis and, if imaging is unhelpful, brain biopsy may be required. The lymphoma is usually a single mass with oedema, but it may be multifocal. Histology shows most PCNSLs to be high grade B-cell neoplasms and there is a very strong association with Epstein-Barr virus, the genome of which can be found in 90% of lymphoma masses and in virtually 100% of PCNSLs in AIDS. Treatment with chemotherapy and /or irradiation is of little avail and life expectancy, like that of PML, is measured in months.

f. HIV-associated dementia (HAD): characterised by global cognitive deterioration and associated motor abnormalities, occurs late in HIV infection, when the CD4 count is generally less than 100cells / cu.mm. Its incidence has fallen considerably since the introduction of HAART. Cerebral atrophy is present and this is detectable on magnetic resonance imaging.

g. Spinal cord involvement: the spinal cord may be affected by a condition called vacuolar myelopathy – similar in symptomatology to subacute combined degeneration of the cord associated with pernicious anaemia but showing, in

addition to demyelination, symmetrical vacuolation. Vitamin B12 levels are normal and no virus is detectable.

h. Peripheral neuropathy: a distal sensory neuropathy develops in 30% of cases of AIDS when the CD4 cell count falls to below 200 cells/cu.mm. Treatment is relatively ineffective and the problem may be compounded by drug related side effects

i. Proximal myopathy: muscle disease occurs in the later stages of HIV/AIDS. A specific myopathy can develop, but skeletal muscle may also be affected by toxoplasmosis, lymphoma and Kaposi's sarcoma. A drug-related myopathy occurs with zidovudine.

AIDS Related Neoplasms

a. Kaposi's sarcoma: this once rare tumour, which was seen only in a few restricted populations such as transplant recipients and young adults in Sub-Saharan Africa, came into prominence with the initial wave of AIDS sufferers in 1981. It has since appeared to segregate in certain sub-sections of HIV-infected individuals, namely male homosexuals and bisexuals, but also in sub-Saharan Africa amongst the general HIV infected population. Epidemiological studies suggested a transmissible agent and a novel herpes virus, human herpes virus 8 (HHV8), was identified as the responsible organism (Chang et al. 1994). The initial lesion is often a purple skin patch, which then enlarges and forms a plaque and later becomes nodular. Any internal organ may be involved and the pace of the illness is often determined by co-existing pathology. The tumour has a rather variable histological appearance with

endothelial cells, fibroblasts and spindle cells arranged in sheets, in the dermis and subcutaneous tissues. Frequent mitoses are seen in the more aggressive nodular types. Combination therapy has reduced the incidence of the disease and may, in previously untreated cases, result in regression. Simple excision, radiotherapy and chemotherapy all have a part to play in treatment.

b. HIV-associated lymphoma: including PCNSL. The risk of developing both Hodgkin and non-Hodgkin lymphoma in HIV infection is increased and correlates with the degree of immunosuppression, most presenting when the CD4 cell count is about 50 cells / cu.mm. It is the AIDS-defining condition in 5-10% in some cohorts. HAART is considered to have effected a 40% reduction in incidence. Standard chemotherapy is used with a remission rate of about 50%.

c. Ano-genital neoplasms: anogenital cancer is more frequent in HIV infected people and the risk is associated with increased incidence of human papilloma virus infection.

d. Invasive cervical carcinoma: this is also an AIDS-defining condition.

1.8 - Normal Brain Constituents.

Neurons

The neuronal population, which resides in the grey matter, is diverse both in terms of its distribution and the morphology of its individual cell subsets. An example of this is in the cerebellar cortex, where the small relatively featureless granular cells are seen side-by-side with the large distinctive

Purkinje cells. In humans, neurons are estimated to number 100 to 200 billion and they vary in size from 4μ (e.g. the smallest granular cells of the cerebellum) to 140μ in diameter (e.g. the largest motor neurons of the spinal cord).

Each neuron has a cell body, termed the perikaryon, and a number of processes known as dendrites or axons. The axon is generally single, larger and longer than the dendrites and is dealt with separately below.

The nucleus is generally centrally located and its size varies according to the cell size, from 3μ to 18μ. A darkly staining nucleolus serves to identify the larger neurons. The cytoplasm is basophilic, and with appropriate staining, and under the light microscope can be shown to contain neurofibrils and deeply staining granules known as Nissl substance. These were identified under the electron microscope as clusters of endoplasmic reticulum. The dendrites appear as simple extensions of the cell body and branch extensively within the immediate vicinity of the cell.

The function of the neuron, very simply defined, is to do with the receipt of information, its processing or retention and its transmission. Input and output are achieved through two modalities – electrical and chemical. The details of action potentials and their propagation are not considered here, but in the resting neuron the interior of the neuron is maintained at 70-90 mV negative potential with respect to the extracellular space. In activity ionic shift occurs with the entry of sodium ions and an action potential is generated. The propagation of action potentials is along the axon and the rate depends on the diameter and thickness of the myelin sheath, which may attain a velocity of

120 metres /sec. The dendrites are part of the afferent system of the cell and form receptor sites with neighbouring synapses and adjacent neuronal cell membranes.

The axon arises at a special site in the perikaryon, a dome-shaped prominence known as the axon hillock, and which is completely devoid of Nissl substance, as is the axon itself, in contrast to the dendrites. The axon contains mitochondria, neurofilaments and microtubules. Axons over 1μ in diameter are usually myelinated (see oligodendrocyte section below) and may attain a length of a metre or more in the human – from the Pyramidal cells of the cerebral cortex to the lumbar cord – and of course much longer in other animals, such as the giraffe! At their distal ends the axons of the CNS form synaptic contact with other neurons, as with the anterior horn cells of the spinal cord. In addition to the conduction of impulses the axon is responsible for the elaboration and release of neurotransmitters. Many of these are produced in the synaptic terminals of the axons, but the enzymes and other molecules required for their synthesis are made in the cell body and conveyed along the axon by a process called axoplasmic transport at a rate which varies, fast transport at a rate of 400mm/day and slow at 1-5mm/day. Apart from the neurotransmitters and their components, other particles are also carried including membrane-bound vesicles and, in a retrograde direction, metabolites and, in pathological settings, viruses, such as the herpes viruses and rabies virus.

The Macroglia – oligodendrocytes, astrocytes and ependyma

In the CNS, as opposed to other tissues and organs, there is no connective tissue, and early investigators tended to view the non-neuronal elements simply as a supporting framework, calling them neuroglia – the nerve glue of Virchow (Rudolf Virchow, German Pathologist 1821-1902) It soon became clear, through the detailed studies of Ramon y Cajal (Santiago Ramon y Cajal, Spanish neurohistologist 1852 -1934) and others, and more recently from electron microscopy and molecular biology, that the glia were very important functional elements. Numerically the glia outnumber the neurons by 10 to 50 times depending on the region, and are estimated to constitute about half the volume of the human brain.

a. Oligodendrocytes: named because they were seen to have fewer processes than astrocytes - are considered to be the most numerous glial cell in white matter, contributing about 80% of the total. In routinely stained sections they appear as round nuclei, less dense and smaller than astrocytes, and cytoplasm is not generally apparent. Three classes are recognized on the basis of nuclear morphology under the light and electron microscope – dark with nuclear size 3.5-5μ, medium of nuclear size 4-7μ and light of nuclear size 6-8.5μ (Mori and Leblond 1970).

They may also be classified according to their location. They are found in the grey matter closely associated with neurons and are termed perineuronal satellite cells. In the white matter they are disposed in rows along the fibres and are known as interfascicular cells.

Oligodendrocytes are responsible for the formation and maintenance of myelin. The process is complex and involves the encirclement of segments of adjacent axons by tongues of cell membrane, and further coiling, so that a multilayered section is produced – the internodal segment or internode, the typical length of which is approximately 200μ.

The short gaps between adjoining segments are known as nodes of Ranvier and are 0.8-1.1μ in length. A single oligodendrocyte may contribute segments to as many as fifty axons and the final thickness of each segment depends on the diameter of the axon. Myelin in its fresh state appears white, and at ultrastructural level the sheath is seen to be composed of alternate concentric layers of lipid and protein.

Myelination correlates directly with brain volume and its progress may be inferred by the facts that at birth the brain is approximately 25% of its volume in adult life and by the end of the first year it has reached 75% of the final adult volume, which is reckoned to occur in the seventeenth or eighteenth year of life. Different tracts myelinate at different times and detailed post-mortem studies have provided data that illustrate this variation (Brody et al. 1987). At birth myelination in the cerebellum was well advanced. At the end of the second post-natal year though myelination was still found to be incomplete in the spinal cord the majority of cerebral white matter tracts were almost completely myelinated including the internal capsule and the temporal lobe white matter. Currently MRI scanning is contributing to our knowledge of white matter maturation, which is shown to continue throughout childhood and adolescence (Paus et al. 2001).

Oligodendrocytes express the following molecules – galactocerebroside (gal c), carbonic anhydrase 11 (CA11), myelin basic protein (MBP) and transferrin. Antibodies to these may be used in immunocytochemistry to aid their identification but a fully comprehensive marker awaits discovery.

Oligodendrocytes react to a wide range of metabolic and toxic insults by acute swelling. A sub-set in adult life retains a limited capacity for proliferation, as is seen in the periphery of MS lesions (Morris et al. 1994).

b. Astrocytes are the largest of the glial cells, having a perikaryon of 10-20μ, and a nucleus of approximately 10μ, in diameter. In number they constitute 10-15% of the total glial population of white matter. Under the light microscope and in routinely stained sections their cytoplasm is not usually visible, and in normal white matter the only clues to their identity are nuclear size and dispersed nuclear chromatin. In the early days of silver staining in neurohistology the astrocytes in grey matter were distinguished from those in white matter by having fewer and shorter processes. The former were termed protoplasmic, and the latter fibrous astrocytes – the name of the cell referring to its stellate shape. Both fibrous and protoplasmic astrocytes contribute to the almost complete covering of the capillary by their end feet (see BBB below). Both likewise participate in the formation of the CNS surface covering and interface with the pia, its basal lamina and their end-feet or cell bodies – the glia limitans.

The fibrils first noted in silver stains appear at ultrastructural level to be hollow structures, which are about 10mm. in diameter and bearing some resemblance to neurofilaments. They occur in both the perikaryon and

processes as compact bundles, and their main constituent is an intermediate filament protein known as glial fibrillary acidic protein (GFAP) that has a molecular weight of 27.000 kD. They are found in protoplasmic astrocytes as well but are far fewer in number. Astrocytes have a capacity to change rapidly in morphology that occurs without alteration in the cytoskeletal proteins (Safavi-Abbasi et al. 2001) (cell culture study).

Since 1972 GFAP has been used as a marker for astrocytes (Bignami et al. 1972) using polyclonal, and now monoclonal, antibodies. There has been general acceptance that resting protoplasmic astrocytes do not show significant staining and the fibrous astrocytes of young animals are also less well stained. Furthermore in the adult animal a proportion of cells having astrocytic morphology remain unstained.

Astrocytes perform a number of functions in health. The idea of the early histologists that the main purpose of astrocytes was to provide support for the neuronal population is inseparable from what is now known of the micro-architecture of the astroglia – their attachment to the fine vessels of the neuropil and their formation of the glia limitans. Astrocytes play an essential part in the induction and maintenance of the Blood Brain Barrier (Hayashi et al. 1997). In embryogenesis they provide a framework for migrating neuroblasts. Ultra structural studies have revealed that almost all synapses are covered by the fine processes of astrocytes (Nedergaard et al. 2003). Protoplasmic astrocytes are known to absorb neurotransmitters from the synaptic cleft (Anderson and Swanson 2000). Another important function is the uptake of extra-cellular potassium (Walz 2000). Astrocytes are involved

in energy metabolism and store glycogen (Forsyth 1996), but the precise mechanism of glucose transport from blood to neuron and the role of the protoplasmic atrocity remains incompletely explained.

A wide variety of conditions are associated with a rapid change in astrocytic morphology and function. An early and easily recognisable change seen in routinely stained sections under the light microscope is swelling of the cell body, with abundant eosinophilic cytoplasm and displacement of the nucleus. This change is seen in response to generalised conditions such as hypoxia, or local ones such as in the proximity of tumours or acute multiple sclerosis (MS) lesions. If widespread and severe the problem of cytotoxic brain oedema may arise, with its metabolic and pressure effects. A more profound change, which may be associated with any conditions causing acute swelling, is known as **astrogliosis** (Eng and Ghirnikar 1994). This may be defined as an acute reactive state of astrocytes in which there is both hypertrophy, as evidenced by an increased cell GFAP content, and hyperplasia. However, research has suggested that in the acute reaction the impression of an increase in numbers of astrocytes is actually due to the upregulation of GFAP in previously GFAP negative cells, rather than local proliferation or migration of nearby astrocytes (Eddleston and Mucke 1993).

c. The **ependyma** is a single layer of cuboidal cells lining the central canal of the spinal cord and the ventricles of the brain. The cells have microvilli on their apical surfaces. Their basal processes, which extended in the embryo to the surface of the neuraxis, in postembryonic life remain as vestigial remnants, which intertwine with a layer of astrocytes to form the sub-ependymal or

internal limiting glial membrane. In contrast to most other epithelia, no basement membrane is present. The ependymal cells are joined to each other by the usual epithelial junctional complexes and there is no functional barrier, akin to the blood brain barrier (BBB), between the CSF and the brain. Indeed the ependyma is believed to have absorptive and secretory functions. Ependymal cells in the adult have no regenerative capacity.

The Microglia and Perivascular Cells

These two cell types are both considered to be of monocyte/macrocyte lineage.

There is general acceptance that the microglia in the mature nervous system are directly derived from a population of monocytes which moved into the foetal nervous system, adopted a universal, but not necessarily uniform, distribution, lost their amoeboid nature and became the typical resting ramified cells in the neuropil.

The terminology of the perivascular macrophages or perivascular cells remains the subject of debate, but certain features separate them, in their resting state, from the microglia and any other vessel-associated cells. In position they are outside the parenchyma of the CNS, being situated between the basal lamina of the endothelial cells in capillaries or the adventitia of larger vessels and the glia limitans of the parenchyma (Graeber et al. 1992). They are relatively large, lack the dendritic morphology of the microglia and express antigens not detectable on resting microglia (Franklin et al. 1986). It is thought that monocytes entering the nervous system are not eligible to become

microglia but, experimentally, blood monocytes may adopt a microglial morphology when cultured with astrocytes (Sievers et al. 1994).

Resting microglia have a very low rate of population turnover – less than 1% over nine months and longer- but perivascular cells may cycle up to 30% of their population over three months (Hickey et al. 1992). This may play an important part in HIV entry.

In terms of distribution microglia are arranged in the brain and spinal cord in close proximity to one another but without overlap of their spatial domains. They constitute some 5-10% of the total glial population and are probably found in equal numbers in grey and white matter. Microglia, in their resting or ramified state, are difficult to identify with certainty in routinely stained sections under the light microscope, but generally the nucleus is round, dense and small - between 5-8μ in size. Staining with heavy metals shows that the cell body is smaller than the other glia, is bipolar, and has two or three branches extending from either end, each of which is subdivided into finer branches.

The most effective immunostains for the microglia are anti-CD 68, anti HLA–DR and anti-CD 45, and for the perivascular cells, anti-CD 14 and anti-CD 45. The state of activation, as indicated by cell surface antigens, does not necessarily correlate with morphology, i.e. whether the cell is in its ramified or amoeboid state (Dickson et al. 1991) .

Though the microglia show a down-regulated phenotype with ramified morphology in the CNS of healthy animals, they are the first cell to respond to

even subtle molecular signals, either locally, or as a manifestation of minor and remote systemic disease (Perry et al. 1993), (Perry 2007).

Microglia when activated, apart from their phagocytic function, also produce numerous molecules, some of which are potentially cytotoxic. Amongst these are nitric oxide (NO), free oxygen intermediates, proteases, excitatory amino acids and cytokines. The involvement of some of these is considered later in this section, but here it may be noted that the pro-inflammatory cytokine TNF-α may cause damage in multiple sclerosis, and free oxygen radicals have a neurotoxic effect on neurons in co-cultures (Thery et al. 1991). Apart from their role as cytotoxic effector cells, microglia may exert a protective influence in reducing post-inflammatory gliosis by secretion of TGF-β1.

Choroid Plexus

The choroid plexus is a highly vascular frond-like structure in four parts, located in the lateral ventricles, the third ventricle and the fourth ventricle. It arises in the embryo as an invagination of the ependymal roof plate into the ventricular cavities by the pial blood vessels. Its function is the production of the CSF, both by ultra-filtration and secretion.

It is produced at the rate of approximately 0.35mls /min. and measures in volume in the adult 140 mls. A blood-CSF barrier exists in the same way as the blood brain barrier and is located at the cuboidal epithelium that covers the surface of the choroid plexus. Tight junctions join these cells.

Vascular Endothelium

The Vascular endothelium of the CNS plays an important part in the pathogenesis of many diseases affecting the brain and spinal cord. As it is of a special nature, some detail of normal capillary structure and function is included here. The typical capillary wall is a single layer of the endothelial cells (which line the entire vascular system), arranged on a basal lamina. Other cells called pericytes are associated with capillaries. The capillary diameter is 5-8μ, allowing blood cells to pass in single file with some transient deformity. Their length is between 200μ and 1 mm. and the pressure within 25mm Hg. The entry to the capillary system is through arterioles, the immediate pre-capillary arteriole having a single layer of muscle cells and contraction of which can deny entry of blood to the capillary network. The exit is provided by the confluence of several capillaries to form a vessel of up to 30μ in diameter, known as a post-capillary venule. This has a thin adventitia of collagen fibres but has no muscle coat or elastic lamina. This is probably (see below) the principal site of leucocyte migration and certainly the chief site of protein rich fluid escape in neurogenic inflammation. The intraluminal pressure drops from 25mm to 5 mm of mercury in these vessels.

The endothelial cells are flat and thin, being 2-3 μ at central nuclear level and 0.2 μ peripherally. The junctions between neighbouring endothelial cells are classified as to the degree of adhesion – adherens, communicans and occludens - of which the last is the most secure and which is found in the almost all of the capillary networks in the CNS. Another specific feature of capillaries in the CNS is that their surface is almost completely enveloped by the end feet of

astrocyte processes. Endothelial cell functions vary in different parts of the body but in general they contain numerous enzyme and transport systems, they secrete clotting factors and express adhesion molecules to attract leucocytes. In the endothelial capillary cells of the CNS the nucleus shows homogenous chromatin. Mitochondria are more numerous than in endothelial cells elsewhere. True pinocytotic vesicles and lysosomes are rare in normal health. CNS endothelial cells can be identified by immuno -cytochemistry, using antibodies to Factor 8 related antigen (Von Willebrand protein) and anti-ZO-1, an antibody to zona occludentes.

The Leptomeninges

The leptomeninges, known as pia mater and the arachnoid mater are the fine layers inside the tough outer dura mater that cover the brain. They are intimately related to each other. The arachnoid, the outer of the two, is a delicate non-vascular membrane that overlies the vessels on the surface of the brain and bridges over the sulci. The pia is a vascular membrane, intimately attached to the surface of the brain and composed of reticular and elastic fibres. On its upper surface it is connected to the arachnoid by trabecular strands and on its under surface it is continuous with the end feet of astrocytes. The CSF circulates in the space between these two membranes, known as the sub-arachnoid space, and is absorbed into the superior sagittal sinus by passing through the arachnoid granuations, glomerular shaped structures that penetrate the dura mater, to enter the sinus. The pia invests arteries which pass into the brain, becoming fenestrated before blending with the vessel at pre- or capillary

level. Such a space as exists between the vessel and pia is continuous with the perivascular space on the surface of the brain.

The pia and arachnoid are mesodermal derivatives and, in contrast to the CNS parenchyma, neutrophil access is unrestricted.

The Blood Brain Barrier (BBB)

Before examination of the detailed dynamics of viral and CNS interactions, the structure and concept of the Blood Brain Barrier (BBB) is considered in some detail. In the context of HIV infection its importance lies in the mode of viral entry, the constraints on immune cell access and restriction on the transfer of some of the major therapeutic agents.

In 1885 Ehrlich (Paul Ehrlich, German medical scientist and pioneer of immunology 1854-1915) observed that certain dyes injected into the blood stream of experimental animals, with a view to determining oxygen consumption, stained all tissue and organs except the brain and spinal cord. The concept of the Blood Brain Barrier developed in the early 1900s in studies on the administration of drugs via the CSF route (Lewandowski 1900) Since then it has been the subject of numerous experiments (Davson and Oldendorf 1967). The understanding now is that the principal barrier is at the levels of the endothelial cells of the cerebral capillaries and the post capillary venules. As opposed to most capillaries elsewhere in the body, the borders between the endothelial cells of the cerebral capillaries are sealed by the extension of tight junctions.

In some special locations of the CNS, the pineal body, the neurohypophysis and the median eminence of the hypothalamus, these are lacking. Also known as zonulae occludentes, tight junctions are present as short segment areas of fusion between the endothelial cells in most capillaries of the body, but intervening clefts are present – gap junctions. The studies of del Rio Hortega (Pio del Rio Hortega, Spanish histologist 1882-1945) had shown that the abluminal surface of CNS capillaries was almost completely covered by astrocyte endfeet.

In health, transport of molecules through the endothelial cell itself is strictly regulated. Lipid solubility, hydrogen bonding and molecular weight are the principal determinants of simple diffusion (Oldendorf 1974). Transport systems exist for most essential substances, including nutrients ((Davson and Oldendorf 1967). In certain disease states, especially those in which hypoxia, toxic chemicals or physical factors are present, the integrity of the endothelial cell is compromised, and passage of normally excluded molecules occurs. This process, known as adsorptive endocytosis (AE), has been studied using molecules such as the toxic glycoprotein, wheatgerm agglutinin (WGA), conjugated with horseradish peroxidase (Broadwell 1993). AE may provide a mechanism whereby viral entry is facilitated (Banks et al. 1999).

Therapeutic attempts using hyperosmotic solutions to "open" the BBB to allow enhanced access of chemotherapeutic agents, as in the treatment of brain tumours, produce multifocal lesions in association with serum protein seepage (Salahuddin et al. 1988).

Current views on immune cell entry are considered below and in the final discussion section – chapter 6.

In summary, the principal functions of the blood brain barrier are:

1. Protection of the brain from the blood milieu
2. Selective transport of various substrates or factors which are required for neuronal or glial acitivites
3. Metabolism or modification of blood- or brain-borne substances (Risau and Wolburg 1990).

1.9 - Immune System and the Brain: Neuroinflammation

Reference to one of John Hunter's observations serves as a reminder that inflammation is a response, not a disease in itself, or even a cause of disease: he wrote, in the introductory chapter to his Treatise on the Blood, Inflammation and Gunshot Wounds, published in 1793:

"Inflammation must have some exciting cause, and the same cause will produce an effect under one circumstance, which it will not under another."

Before considering the immunodynamics of the CNS in detail in health and disease, the concept of immune privilege or immunologically privileged sites is briefly mentioned. In the early days of experimental tissue transplantation it was discovered that grafts of foreign tissue would survive in certain locations in the recipient, whilst being rapidly destroyed by immune reaction in others (Medawar 1948). The brain was found to be one such site, the eye and the testis others. Now the understanding is that this is a relative, rather than an

absolute, privilege and is subject to certain conditions. Though the separate determinants of this exclusiveness are considered below and elsewhere, the most important factors, in respect of the CNS, may be summarised:

1. The presence of the BBB excludes large molecules, for example IgG.
2. Its endothelial cells are specialized in their pattern of cytokine secretion.
3. The CNS is devoid of antigen-presenting cells.
4. The parenchymal cells of the CNS do not express MHC class I and class II molecules.

5 Neurons have immunosuppressive actions on glial cells.

6. Glial cells themselves express immunosuppressive cytokines.

Immune reactions in the CNS can be thought of as innate, associated with changes in microglia and astrocytes, or acquired as the result of movement of cells, lymphocytes and monocytes, into the brain compartment.

The CNS has no lymphatic vessels although drainage occurs in an indirect fashion – see below. Immune cell traffic through the brain is normally low in health. The normal CSF contains fewer than 5 cells/ml, of which 70% are lymphocytes and 30% are macrophages. In infection, the cell count rises to 25-500/ml in mumps meningitis, and to > 10,000 in bacterial meningitis. In chronic inflammatory disease, such as multiple sclerosis (MS), it is seldom more than 40/ml.

In the previous section reference was made to the surveillance function of the immune system, which is in effect a detection and early warning system of change in the tissues, whether due to neoplasia or the presence of foreign

material of any kind. All tissues and organs are continuously patrolled and the brain parenchyma is no exception but the numbers of cells and their transit pace are strictly controlled by a number of factors, most of which operate at BBB level. The physical nature of the interface has long been recognized, but in recent years molecular biology has revealed the subtleties and complexities associated with capillary wall physiology. An example of this is the expression of adhesion molecules necessary for leucocyte migration, and which are normally expressed at low levels (Bart – 2000).

The relationships between the various cells of the immune system and the CNS are now separately considered.

Dendritic Cells

Dendritic cells are not normally found in the brain or spinal cord, but in the rat a very small population may be found in the meninges (Matyszak and Perry 1996), and they may rarely be detected in the choroid plexus (McMenamin 1999). Perivascular cells, microglia and astrocytes can all subserve an antigen-presenting function.

T Lymphocytes

T lymphocytes are known to traverse the normal CNS in very small numbers (Hickey – 1999). They do so in apparently random fashion but activation appears to be a condition of entry (Fritz et al. 2000). The adhesion molecule P-selectin has been shown to be involved in lymphocyte access (Hickey 2000). In the absence of an antigenic encounter, the fate of T4 lymphocytes in the

neuropil is unknown. Apoptotic death has been observed in inflammation (Bauer et al. 1998), and the notion that the neural micro-environment is hostile to lymphocytes has been expressed (Hickey 2001). The probable underlying immunological mechanism to account for this apoptotic fate is the fact that astrocytes and neurons in the rat and human brain express fas ligand (fasL). Fas is an apoptosis-inducing protein which can react with the fas receptor of a trafficking lymphocyte (Bechmann et al. 1999)

B Lymphocytes

The relationship between B-lymphocytes and the CNS in health had been studied in the late 1970s. An animal model system was used to test the responses to antigen, to which the animal was already sensitisedand which was introduced behind an intact BBB through a very fine cannula (Cserr et al. 1978). Antigen-specific B cells and plasma cells were then found around the cannula tip providing evidence of a normal micro-inflammatory response. The question as to whether or not B lymphocytes might be found in normal brains, however, remained unanswered until a post-mortem study in 2003 comparing normal brains with those of several groups of HIV-infected individuals (Anthony et al. 2003). B lymphocytes, bearing the activation marker CD 23, were found in small numbers in all parts of the normal brains. Relatively increased numbers of B cells were detected in the brains of late pre-symptomatic (that is after a course of several years) HIV infected individuals but the numbers were found to be reduced in AIDS cases.

The Monocyte/Microglia/ Macrophage System

Monocytes, which may be up to 20μ. in size, comprise 2-10% of the circulating white blood cells. They are the antecedents of macrophages, which they become on entering the tissues after spending the first few days of their life in the bloodstream.

In the tissues, having become macrophages, two populations are encountered – fixed and free – the former being referred to as histiocytes and sometimes named specially according to their locations by their first describers, e.g. Kuppfer cells of the liver and Langerhans cells in the skin. The macrophage, like the neutrophil, is a motile and actively phagocytic cell.

The process of phagocytosis is complex but in essence it consists of particle engulfment, facilitated if the particle is already coated with a host-derived protein known as an opsonin.

The monocyte-macrophage lives for many months and as some bacteria, in particular pathogenic mycobacteria, have evolved evasion strategies to enable continued life within the cell (Nguyen and Pieters 2005), this presents considerable problems for the host. Equally devastating is the same practice of some viruses, such as HIV, as will be seen below.

Neutrophils are short-lived and rarely enter the brain, though in the meninges rapid and major influxes occur in response to bacterial infection.

Monocytes have been known to enter the perivascular spaces in the brain since rat chimaera studies in 1988 (Hickey and Kimura 1988), there to become known as perivascular cells or perivascular microglia. Parenchymal microglia are similarly derived from blood cells circulating form the bone marrow in

early fetal life. The entry of monocytes into the brain is continuous in the healthy animal but their time of exit, if at all, is uncertain. They are efficient antigen-presenting cells and one study suggested that they did leave the CNS to return to the lymphoid tissues (Broadwell et al. 1994). Other studies using particulate antigen failed to demonstrate an immune response (Matyszak and Perry 1996). The relative stability of the population of perivascular cells suggests they either exit or die by apoptosis since there is no evidence to suggest that they move into parenchymal microglial territory in healthy adult life.

Factors Affecting Immune Cell Access

Cytokine modulation is an important mechanism whereby immune cell access is regulated. An important molecule, which enhances entry, is transforming growth factor-β (TGF-β). This is a homo-dimeric protein widely distributed in mammals, and which controls lymphocyte activation, growth and differentiation. Like IL-10, TGF-β can down regulate lymphocyte activation. It is produced by platelets, endothelial cells, lymphocytes and macrophages. It is normally present in health at low levels in the CSF (Johnson et al. 1992).

Glial Cell Participation in Immune Reactions

In vitro studies have suggested an important possible role in T-cell mediated immune responses. Not only is it considered possible that microglia and astrocytes may act, in addition to the dendritic cells of the meninges and choroid plexus, as antigen presenting cells (Aloisi et al. 2000), but that they

can also stimulate the T helper subsets, Th1 and Th 2. The cells of these subsets operate in contrasting ways. Th1 cells when activated produce predominantly pro-inflammatory cytokines (IL-2, IFN-γ, TNF- α and TNF-β) and Th 2 cells produce IL-4, IL-5, IL-6 and IL10 (Mosmann and Sad 1996). These are associated with responses, respectively, the former to elicit phagocytic defence against infections and to activate CD8 T-cells, and the latter to promote B-cell maturation and antibody production. There is interplay between the two processes and the initiation and outcome depend on signalling from local or remote sources. TGF-β can act in an autocrine fashion suppressing both phagocytic and cytotoxic behaviour (Merrill and Zimmerman 1991)

1.10 - HIV and the Brain – pre-AIDS and AIDS, untreated and treated

Viral Entry into the Brain

The possible routes of entry of HIV into the Central Nervous System (CNS) are of considerable interest, not least because of the immunologically privileged status of the brain (Barker -1977), the variable access to therapy and its role as a sanctuary for the virus. The viruses that are known to infect the CNS gain entry in a variety of ways. Some, such as the picornaviruses (Couderc et al. 1990), and parvoviruses(McClure 1992) infect the capillary endothelial cells and gain entry in this way. Others, for example, coxsackie and mumps virus, infect the meninges. Neural spread is recognized as a route for

rabies virus (Murphy et al. 1973) and offers a conduit for many other virus families including herpesviruses and polioviruses, but does not appear to participate in HIV entry.

An early observation that HIV might infect CNS endothelial cells (Moses et al. 1993) has not been confirmed. This finding, which was for a lymphotrophic strain, would be at variance with the demonstration that in early HIV infection the macrophagetropic HIV is the predominant viral phenotype (Zhu et al. 1993). The choroid plexus has been shown to harbour HIV infection in both asymptomatic and AIDS patients (Petito et al. 1999).

However, the most likely and important route for HIV entry into the CNS is considered to be within blood-derived monocyte/macrophages (Nottet and Gendelman 1995), and its immediate place of residence in the perivascular spaces within the perivascular macrophages (Koenig et al. 1986). The precise molecular mechanisms enabling entry to the CNS remain unclear, but the first step seems to be the rolling of the infected cell on the endothelium. This is mediated by the interaction of the endothelial adhesion molecule E-selectin and oligosaccharides present on the monocytes (Varki 1994). This process is likely to operate later in the AIDS phase as the expression of E-selectin is considerably increased in demented compared with non-demented cases (Nottet et al. 1996). Another factor which may aid entry is the production by HIV-infected cells of NO, a potent vaso-active molecule which could slow the capillary bloodstream.

The possible routes from capillary lumen to perivascular space are still a matter of debate and recent papers examine the matter critically (Engelhardt

and Wolburg 2004), (Bechmann 2006) (and see below - BBB). In essence the cells could migrate in the capillary section, either through the endothelial cell itself, or in between cells in the tight junction region and the astrocyte end feet, or distal to the capillary through the wall of the post-capillary venule.

The fate of the infected perivascular cells after the initial inflammatory reaction of acute HIV infection is debateable; most seem to disappear, either dying or passing out in the CSF. A few remain, but these may not necessarily contribute to the protracted second wave that heralds the onset of pre-AIDS. In this phase there are high levels of circulating cytokines, such as TNF-α and IL-1β, which alter BBB permeability (Quagliarello et al. 1991), and these induce the expression of endothelial adhesion molecules E-selectin and vascular adhesion molecule-1 (ICAM-1) (Nottet et al. 1996). The BBB in this terminal infection is probably further damaged by the monocytes' secretion of super oxide anion and NO, which can combine to form the highly toxic compound perioxynitrite (Boven et al. 1999). An allied compound, nitrotyrosine, can be demonstrated by immunocytochemistry in the perivascular areas of demented AIDS patients. Infected monocytes also have the capacity to digest basement membrane. The factors affecting HIV- infected macrophages' migration within the CNS parenchyma and their preferential location in the cerebral white matter remain unclear, but a spectrum of chemoattractant molecules is likely to be involved (Nottet – 1997), including TGF-1 and macrophage inflammatory protein-α (MIP- 1α), both of which are found in higher levels in AIDS brains compared with controls. An association with astrocytes may be significant.

The Neuropathology of HIV/AIDS

The neuropathology, as related to the stages of the disease and the conditions outlined above, is now considered. The CSF abnormalities already noted suggest that from the primary infection until death, when as many as 90% of patients dying of AIDS will have detectable CNS changes at necropsy (Kure et al. 1991) (Gray et al. 1988), HIV maintains a presence in the CNS which is clinically silent for much of the time.

At the time of the acute primary infection much of what happens in the human brain is speculative, but a single case report (Davis et al. 1992) allows insight into what may be the universal situation. A 68 year- old man, infected as a result a therapeutic error, died two weeks later. Blood culture for HIV-1 had become positive just before death. At necropsy a mild lymphocytic meningitis and a minor degree of perivascular cuffing was present. No focal abnormalities were noted. HIV was obtained by culture and HIV gp41 viral antigen was detected in sparse perivascular and sub-pial cells. Several other organs sampled showed no signs of viral presence. In the light of accumulated knowledge of HIV infection and simian studies (Chakrabarti et al. 1991) it seems reasonable to suppose that this represents the usual state of CNS involvement in the early weeks after infection.

The long asymptomatic stage of HIV infection is marked by continued viral activity in the lymphatic system and CD4 lymphocyte depletion, and opportunities to study other organs are infrequent but are provided by cases of accidental death, related to substance abuse, suicide or haemophiliacs dying of

complications. Several studies have reported the general and CNS pathology in these pre-AIDS individuals. In one study of four cases no significant abnormality was seen (Budka 1991). In others a low grade lymphocytic meninigitis and perivascular infiltrate was observed, but microglial nodules and multinucleate giant cells were not (Esiri et al. 1989) (Bell et al. 1993), (Gray et al. 1996). Astrogliosis and microgliosis were noted. The presence and timing of active viral replication remains uncertain with respect to the pre-AIDS stage (An et al. 1996), but a marker of transition to HIVE may be the increase in B and T cell numbers in the perivascular compartment of the brain (Anthony et al. 2003).

The neuropathology of the final stages of HIV infection – AIDS - was of interest from a very early stage in the epidemic as CNS manifestations such as dementia and motor abnormalities were of frequent occurrence (Snider et al. 1983) , (Jordan et al. 1985)

In 1985 a post mortem study of 15 patients, adults and children, dying with AIDS-related encephalitis showed varying neuropathology (Shaw et al. 1985). In addition to focal lesions, due to CMV or Toxoplasma, other features were cerebral atrophy, scattered microglial nodules and vacuolar myelopathy. One brain appeared normal. Histological abnormalities did not consistently correlate with the degree of dementia. Detailed Southern blot analysis and in situ hybridisation confirmed the presence of HTLV-III DNA in the brains of five of the cases and in four of these viral-specific RNA was found (Shaw et al. 1985), suggesting that the virus itself might play a major part in the genesis of the encephalopathy. It was noted that the features of this condition contrasted

strongly with those of Visna, in which there was a strong mononuclear inflammatory response in the brain but which occurred in the absence of the profound immunosuppression present in HIV/AIDS.

Other studies in the same year, 1985, showed that HIV could be isolated and grown from the CSF and other CNS tissues (Levy et al. 1985).

In 1986, the cellular localisation of HTLV-III (which had just been renamed HIV) was investigated in the brains of twelve AIDS cases (Wiley et al. 1999). Using the technique of in-situ hybridisation, nine of the brains were shown to be significantly infected with HIV and the cells involved were capillary endothelial cells, mononuclear inflammatory cells and giant cells. White matter was more severely affected than grey matter. Only one case, with severe and extensive involvement, showed evidence of weak infection of neurons and astrocytes; the important conclusion drawn was that features of AIDS-related encephalopathy were more likely to be due to an indirect effect of the virus than compromise of neurons or astrocytes by direct infection (Wiley et al. 1986).

In the 20 years that have elapsed since then, many aspects of the neuropathology of HIV/AIDS have been explored, and imaging studies have contributed to our understanding of the extent of the disease process in treated and untreated patients, and those in special risk groups such as drug abusers. The hallmark of productive HIV infection in the CNS remains the presence of HIV positive, macrophage-derived giant cells, signalling the presence of HIV encephalitis (HIVE).

In general the naked eye appearances of the brain may be unremarkable. The reduced brain weight noted in childhood HIV infection (Bell et al. 1997)((Kozlowski et al. 1997) has not been systematically reported for adults. The meninges do not usually show any obvious gross abnormality. A minor degree of gyral atrophy and, on sectioning, some ventricular dilatation may be present. MRI imaging studies confirm these findings in AIDS subjects (Everall et al. 1997).

The changes seen on microscopy vary considerably and there may be no correlation between the appearances and the degree of dementia noted in the months before death. Some parts of the brain are generally affected more than others – the cerebral cortex is less involved than the deep grey matter, and the central white matter of the cerebrum more than that of the cerebellum (Gray et al. 1988). The commonly reported finding of white matter pallor in haematoxylin and eosin or Luxol fast blue stained sections may have as its basis significant white matter pathology with myelin loss and astrocytic activation (Budka et al. 1987). Perhaps more commonly, it may be a reflection of underlying oedema, or even an artefact related to poor fixation. White matter pathology was originally considered to be of two distinct kinds – HIVE, in which there were multi-focal infiltrations of inflammatory cells with only minor focal areas of myelin damage, and HIV leuco-encephalopathy (HIVL), which was characterised by diffuse or focal loss of myelin, sometimes with axonal loss as well, and in severe cases, frank necrosis. Associated with this latter change was microglial/macrophage infiltration and astrocytic activation. Now the justification for considering these two conditions as separate entities

seems less, and it is supposed that they may represent different stages on a continuous spectrum of white matter damage (Grassi et al. 1997).

The cellular elements respond in different ways to HIV infection, and it is not always clear whether they are reacting to local or remote influences.

Neuronal loss, initially sought as a cause of, or correlation with, HAD is generally quite modest (Everall et al. 1993) and may not be detectable (Seilhean et al. 1993) Although HIV-specific nucleic acid has been demonstrated in neurons by in situ polymerase chain reaction (in-situ PCR), productive infection has not been demonstrated in these cells.

Astrocytes respond to HIV infection, as they do to most environmental perturbations in the CNS, by upregulation of GFAP and proliferation. Though they lack the surface receptor CD4 they have been shown to become infected in small numbers (Epstein et al. 1984), and have been shown to support productive infection in tissue culture (Brack-Werner 1999). Productive infection of astrocytes in vivo is considered to be limited (Saito et al. 1994); but more important are the possibilities of associated malfunction of important homeostatic functions of this cell, including maintenance of the BBB, and its potential capacity as a reservoir of the virus.

The role of the oligodendroctye in HIV infection is uncertain. It is not known to become infected, but certainly participates in the general white matter disturbance. Its numbers were found to be increased in HIVL (Esiri et al. 1991). The damage to myelin may be due to a direct effect of toxic molecules, or an indirect one through the agency of the oligodendrocyte.

Macrophages, giant cells and microglia play the principal part in the pathogenesis of HIVE. All of these cells have been shown by immunocytochemistry to be productively infected (Budka 1990). The sequence of infection of these cells and their relative numbers is probably not as important as the relationship between the number of giant cells and the proviral load (Bell et al. 1996) Giant cells are thought to originate from fusion of infected macrophages (Michaels et al. 1988; Moses et al. 1993). Microglial nodules are common in HIVE but are non-specific (Kato et al. 1987). They are found more commonly in the white matter.

There is a difference in the neuropathology between different risk groups. Haemophiliacs show less advanced change associated with HIVE and fewer opportunistic infections (Esiri et al. 1989). They are however more prone to suffer from cerebral haemorrhage.

HIV infected drug misusers were shown to have a higher incidence of HIVE than homosexual men, but the reverse relationship was found with respect to primary cerebral lymphoma (Bell 1998).

The Neurological Syndromes Associated with HIV Infection

Involvement of the CNS in HIV infection, considered in part in the beginning of this section, was common in the early years of the epidemic. Some 39% of AIDS patients had neurological abnormalities (Levy et al. 1985). Even in the absence of symptoms or of recognisable signs, examination of the CSF may show elevated protein levels, pleocytosis and the presence of viral RNA as well as anti-HIV antibodies.

In adults (for children see section 2.5) the main clinical syndromes are:

- Meningitic syndrome
- Seizure disorder
- Motor and sensory disorders
- Cognitive disorder
- Ophthalmic Disease (with visual compromise)

Meningitic syndrome: this syndrome occurs in the course of the primary infection in about 10% of cases (Carne et al. 1985) (Schacker et al. 1996). It may be accompanied by a rash and lymphadenopathy and lasts 2-4 weeks. It is indistinguishable on clinical grounds from other causes of aseptic meningitis, but HIV-RNA can be found in blood and CSF.

It can also develop at any other time prior to the onset of AIDS, and the fact it is very uncommon in AIDS is thought to signify that it may have an immune basis (Fauci 2001).

Seizure disorder: although seizures may occur occasionally in the meningitic illness of primary infection, most instances occur in AIDS either as a manifestation of direct HIV infection, such as sub-acute encephalitis (7-50%), or in the course of secondary CNS disease, for example cerebral toxoplasmosis (15-40%). When a seizure is the presenting symptom of HIV infection, an underlying mass lesion is the commonest cause, usually due to toxoplasma infection or to lymphoma.

Motor and sensory disorders: these are principally determined by disease in the spinal cord, and to a lesser extent, in the spinal nerve roots and peripheral nerves. Spinal cord involvement is confirmed by finding degeneration of the

dorsal and lateral columns on histological examination – a condition known as vacuolar myelopathy. This is found in 5-30% of AIDS cases (Petito et al. 1986) (Henin et al. 1992). The vacuoles, which are symmetrically disposed, appear at ultrastructural level to be intramyelinic swellings with splitting of the lamellae. Generally axons are preserved, except in association with Wallerian degeneration secondary to severe vacuolation. The condition is maximal in the thoracic cord. There is no gliosis and/or inflammatory infiltrate apart from some debris containing macrophages. Viral antigen is not generally found and, if it is, the more correct appellation would be myelitis (Henin et al. 1992). As previously noted, though the condition resembles sub-acute combined degeneration of the cord, vitamin B12 levels are normal.

Cognitive disorder: Soon after the recognition of AIDS in 1981 it became apparent that there was an associated dementing illness, and that this could be the presenting condition in some 3% of AIDS sufferers. In the terminal stages of the disease it is present in at least 25% (Fauci 2008). Originally this condition was termed "sub-acute encephalitis" then subsequently HIV-associated dementia, AIDS dementia complex and most recently HIV-associated major cognitive/motor disorder. AIDS dementia complex (ADC) is currently used most commonly and is taken to be synonymous with HAD.

Its onset is insidious, with mental slowing, apathy, difficulty in concentration and poor memory. Psychological testing has suggested that the onset of HAD in AIDS corresponds to a CD4 count of below 350/cu.mm (Harrison et al. 1998). Features such as dysphasia and dyspraxia, which are common in Alzheimer type dementia, are infrequent, as is impairment of conscious level.

Motor and behavioural problems develop in the course of the condition, which, if untreated, lasts less than a year.

CSF findings show a raised protein level and cell count. HIV-RNA and specific antibodies can be detected, and the virus itself cultured, but there is no correlation between this and the presence of HAD. A spectrum of molecules is also present, including neopterin and quinoleic acid, in addition to inflammatory cytokines and these are all suspected of being pathogenic for CNS damage, in addition to HIV itself.

Imaging by CT-scan or MRI helps to exclude the presence of additional pathology - for example toxoplasmosis or lymphoma - and generally shows a degree of cerebral atrophy. Treatment with HAART usually results in a rapid improvement in cognitive function, suggesting that structural change is not the sole basis for cognitive decline.

Ophthalmic conditions: evidence of eye involvement is found in about half of the cases with advanced AIDS. CMV retinitis is a serious cause of visual loss and occurs when the CD4 count falls below 100/cu.mm. Intensive treatment with intravenous anti-retroviral therapy is mandatory to prevent impairment of vision. Toxoplasmosis and lymphoma may less commonly affect the retina.

1.11 - HIV/AIDS – treatment

In 1981, the first sufferers of what was to become known as HIV/AIDS were afflicted by illnesses symptomatic of underlying severe immunodeficiency,

including cryptococcal meningitis, Kaposi's sarcoma and Pneumocysts carinii pneumonia. Initial treatment was of necessity directed towards these conditions. The life expectancy of such patients was approximately 2 years (Rothenberg et al. 1987) and little therapeutic yield was achieved. In 1983, the identification of the virus, HIV (Barre-Sinoussi et al. 1983), and the later introduction of an antibody test (Silberner 1985), paved the way for the development and approval of the first anti-HIV drug, zidovudine (AZT). This preparation exploited the early stage of HIV entry, where the viral RNA is transformed by the enzyme reverse transcriptase to double-stranded DNA prior to its incorporation into the host DNA. Zidovudine is a nucleotide analogue which inhibits reverse transcriptase (Ezzell 1987) (Furman and Barry 1988). Another nucleotide reverse transcriptase inhibitor (NRTI), didanosine (DDI), was released in 1991 but by 1994 it was recognized that this form of treatment was insufficient (Volberding et al. 1995) on its own to halt the progression of HIV infection to AIDS.

However, also in 1994, a significant success associated with zidovudine therapy was recorded, and that was a reduction in the rate of mother-to-child-transmission from a quarter to just under a tenth of cases (Connor et al. 1994).

The next development in anti-HIV therapy was an intervention in a later stage of intra-cellular life history of the virus – that of protein assembly in the viral particle in its pre-exit maturation phase. Core protein chains at this point are shortened by enzymatic cleavage involving viral protease, inhibition of which precludes maturation to infectious virions (Venaud et al. 1992) (Dorsey et al. 1994). Drugs such as indinavir and ritonavir were approved for general

treatment in 1996. It soon became apparent that, when protease inhibitors were used alone or intermittently, drug resistance emerged in a matter of months. Trials of different drugs given together then followed, and a combination of two reverse transcriptase inhibitors and a protease inhibitor was found to be particularly effective. An alternative approach was to use another type of drug of a class called non-nucleoside reverse transcriptase inhibitors (NNRTIs), of which vivirapine, introduced in 1996, is an example, in combination with an NRTI and a protease inhibitor.

This form of treatment became known as highly active anti-retroviral therapy, or HAART, and suppression of plasma viral load, as measured by RT-PCR for HIV-1 RNA, was shown to remain below detectable limits (Gulick et al. 2000).

HAART is still the mainstay of therapy. Other drugs, such as fusion inhibitors, have been developed. The quest for a vaccine continues and the pessimistic view in 2008 is that an effective agent is still at least 10 years away.

The problems of drug resistance, especially in view of the pronounced tendency of the virus to genetic variation, and the side effects of these drugs, are encountered in therapy, as with other anti-microbial agents. Generally the search for anti-HIV drugs has been constrained by the fact that the virus uses many host molecules, which cannot be used as targets, and current therapy is effective only whilst the virus is replicating. Latent infection in lymphocytes (Wong et al. 1997) and monocyte/macrophage reservoirs (Pomerantz 2001) remain a major obstacle in attempts to eliminate virus by drugs. Furthermore

the drug levels achieved in the CSF may be sub-optimal, e.g DDI concentration in the CSF is only about 20% of that in plasma and the presumption that plasma levels reflects those in brain tissue may be false.

In summary, treatment for HIV has been surprisingly effective in transforming a uniformly fatal disease to a chronic illness but there are still many limitations, some outlined above. However the principal concerns on a global scale are those of accessibility and compliance in poor countries, especially in parts of Africa.

The HAART era

Since 1995, when HAART was introduced, the lives of most HIV sufferers in the Western world have been transformed, and for them the prospect is that of living with a chronic disease rather than a uniformly fatal one. However, for the majority in the developing world the situation remains bleak, especially in respect of the dependency and suffering imposed by the devastating impact of the CNS involvement by HIV/AIDS. The success of HAART in most recipients has tended to obscure certain limitations that are now briefly considered.

For some, treatment is started after a degree of neural damage has occurred and this persists. In others, side effects of therapy dictate cessation, and in yet others a paradoxical and serious worsening of cerebral symptomatology occurs, for reasons not yet well understood. Moreover there is the increasing problem of the emergence of drug-resistant strains. Also, the limited penetration of protease inhibitors into the CNS and viral reservoirs are as yet

unresolved issues. The failure rate in therapy may be as high as 50% (Fatkenheuer et al. 1997). The overall yield is a 50% decline in AIDS death rate, decreased maternal-infant transmission rates, reductions in the rates of opportunistic infections and a 40-50% decrease in the incidence of HIV-associated dementia (Brodt et al. 1997) (Sacktor et al. 2001).

However, the incidence of minor cognitive-motor disorder remained unchanged with HAART, and the prevalence was recorded as running at approximately 30% in two major cohorts of high risk individuals (Sacktor et al. 2002). This was a disappointing finding especially as CSF studies showed effective clearance of HIV-1 RNA over a three month period (Foudraine et al. 1998).

As regards post-mortem findings in the HAART era, the incidence of opportunistic infections has declined (Mamidi et al. 2002), but HIVE was found in 25% of cases in one autopsy study (Masliah et al. 2000). In another study apparently inactive HIV encephalitis (HIVE) lesions were found, suggesting at least temporary respite from disease progression (Gray et al. 2003). A rather different situation has been reported in some cases in the last few years. This has occurred in patients receiving HAART with established neurological disease and who died with worsening symptoms. In a small study of seven cases, post mortem examination revealed active and extensive demyelinating leucoencephalopathy associated with marked perivascular inflammatory cell infiltrates (Langford et al. 2002). A more recent report on two patients with advanced HIV infection and who, under treatment, had shown rising CD4 cell counts and falling viral loads, revealed that both died

with acute encephalopathies. Detailed post mortem examination showed in one, HIVE, and in both, widespread microglial hyperplasia and very extensive perivascular and parenchymal infiltration with CD8+/CD4- lymphocytes. HIV-RNA was detectable by PCR in both brains (Miller et al. 2004).

The explanation offered for the findings in these cases is that the abrupt and selective recovery of the CD8 lymphocyte population in the periphery allows a surge into the relatively unprotected CNS and profound tissue damage follows. This phenomenon of HAART-related adverse immune response has been termed the immune reconstitution inflammatory syndrome (IRIS).

A very important aspect in the development of HIV-associated dementia is the question of the final common pathway – would it develop irrespective of conditions prevailing elsewhere in the body, or is the "driver" in the final phase resident in a locus such as the bone marrow (Gartner 2000), and thereby offering hope of prevention by new systemic treatments?

1.12 - Paediatric AIDS

1.12.1 The General Western Population - with reference to that in the developing world

HIV/AIDS in Children

The upper age limit of childhood is variously defined, but for the purposes of this study the term "children" is used to apply to those under the age of 15 years.

The first cases of AIDS in childhood were reported in 1982, in the USA, at varying locations from San Francisco to New York. By 1996 it had become the seventh leading cause of mortality in the 1-4 years age group in the USA (Ventura et al. 1997)

Now it is estimated that there are 1,500 new infections daily worldwide and 2.1 million children are currently infected. The number of children estimated to have died worldwide in 2007 is 290,000 (270,000 -320,000) (UNAIDS/WHO 2007).

Also of note is that in resource-poor countries, where the major mode of spread in adults is by heterosexual intercourse, a consequence is that some 15-20% of all new cases are paediatric (Kuhn and Stein 1995).

In the worst affected countries AIDS is now the biggest single cause of death in the under 5 age group. The prognosis for African children infected perinatally with HIV-1 is very poor. In a meta-analysis of seven randomized mother-to-child-transmission intervention trials, 32% had died by one year and 52.5% by two years (Newell et al. 2004).

Modes of Transmission

In the first report of immuno-compromised children in the USA, the social and sexual habits of their mothers raised the possibility of a vertically transmissible agent (Oleske et al. 1983). Following the discovery of HIV, its detection in amniotic fluid and products of conception made this mode of transmission almost certain. The further isolation of the virus in breast milk and vaginal secretions pointed to additional routes of perinatal infection. By

1987, when it was already known that the virus could also be spread by contaminated blood or blood products, it was estimated that of the population of childhood AIDS in the USA, some 80% was due to vertical transmission, and the remaining 20% to transfusion of blood or blood products (Rogers et al. 1987). Now, since screening of blood and blood products is universal in developed countries, almost all childhood HIV infection is due to mother–to-child-transmission (MTCT). In less fortunate countries, such as those in Africa, infection by contaminated blood or syringes still occurs and by another mode, particularly in the case of young girls, that of sexual abuse (WHO 2007).

Mother-to-child (MTCT) or Vertical Transmission

Estimates for rates of MTCT in the absence of any therapeutic intervention vary, but reasonably reliable figures are 15-20% in Europe and the USA, and up to 25-30% in some African populations (Prendergast et al. 2007). The transmission rate for HIV-2, even without any intervention, is much lower – less than 2% (Adjorlolo-Johnson et al. 1994) underscoring the limited pathogenicity of HIV-2 compared with HIV-1. A large number of factors influence the risk of vertical transmission. The more important of these include maternal viral load and CD4 count (Burns et al. 1997), prematurity, duration of rupture of the membranes and mode of delivery.

The timing of transmission has been the subject of many studies and differing views are held as to the relative importance of the various time "windows" - pre-confinement, intranatal or perinatal and post-natal. In a study

of spontaneous abortion in HIV positive women, HIV-1 was found in 7 out of 14 foetuses by in-situ hybridisation (Langston et al. 1995). Thus, though transmission may occur in the early weeks of pregnancy, evidence from prospective longitudinal studies suggests that the most important time period is either in the short period before, or actually during, birth and this may amount to as much as 80-90% in developed countries. The studies, which involved estimation of HIV-RNA levels at birth and after, showed that only 12-16% of infected infants were positive at birth, which is consistent with intra-uterine transmission (Shearer et al. 1997). Furthermore, the RNA levels in such positive neonates tended to rise rapidly in the first 2-4 weeks of life, signifying possible recent intra-uterine infection. Another study, based on perinatal virology and serology, estimated that of those considered to have been infected in utero, the median time between infection and delivery was 14 days (95% CI 5-75%), and the 95th percentile was estimated as 59 days before delivery (Rouzioux et al. 1995).

The post-natal transmission risk, that is the risk associated with breast feeding, was accurately defined in African women who became infected in the post partum period and were shown to transmit infection in their milk at rates of up to 30% (Van de Perre et al. 1992). The overall risk from breast feeding in the transmission of HIV is estimated from a meta-analysis to be in the range from 7-22% (Dunn et al. 1992).

The time periods of probable transmission in African populations have been studied, and for general transmission rates of 25% the proportions have been estimated at: 20-25% in the early intra-uterine period, i.e. before 28 weeks of

gestation; 50-60% in the period from 28 weeks to 7 days post-partum; and 15 - 20% in the post-natal period to 24 months (Simonon et al. 1994). The results of this and other studies were in accordance with the study quoted above (Dunn et al. 1992).

Another important factor influencing MTCT is a genetic one – mother-child class 1 HLA concordance increases the risk (MacDonald et al. 1998).

Rates of mother to child transmission are much higher in sub-Saharan Africa than in Europe (Kuhn and Stein 1995), and several factors are probably responsible for this disparity, for example the higher the maternal viral load, the more likely the possibility of transmission (Kamara et al. 2005). Co-existent maternal disease, such as malaria in sub-Saharan African populations, is also known to increase the risk (Brahmbhatt et al. 2008)

Modulation of MTCT Risk by Intervention

In Europe and the USA a number of interventions are available which have been shown to reduce the risk of transmission substantially. Zidovudine therapy given before 28 weeks' gestation can minimize the risk of intra-uterine infection (Lallemant et al. 2000). In the absence of breast feeding, zidovudine given during pregnancy and labour, and to the newborn infant for 6 weeks, can reduce the transmission rate from 25 to 8% (Connor et al. 1994). In women on triple therapy with an undetectable viral load the risk may be as low as 1% (MMWR – 1998). Delivery by Caesarian section, which is known to confer significant benefits in reduction of transmission (Kind 1995), may be indicated in some circumstances In less favoured settings the options are fewer, but

zidovudine in short courses, or nevirapine given as a single dose to the mother in labour and to the infant during the first three days of life, have reduced transmission rates by up to 40%. This has been shown to be a very cost effective intervention (Sweat et al. 2004).

The question of advice on breast feeding for mothers in developing countries is often problematic, and from a pragmatic point of view the infant may be at less overall risk from its mother's milk than other risks such as malnutrition or gastroenteritis from contaminated water supplies.

One simple and inexpensive intervention is the provision of vitamin A supplements for pregnant women. Vitamin A deficiency is common in African populations and is a recognized risk factor for MTCT (Semba et al. 1994).

In considering the impressive advances made in the developing world in the recognition and prevention of MTCT, mention should be made of a parallel adverse situation in respect of vertically transmitted syphilis, another sexually related disease,. In Europe and the USA, antenatal screening for this condition has been standard for fifty years or more, and though there has been a slight increase in reported cases in the general population in recent years, congenital syphilis remains a rarity. However, the position with respect to prevalence and incidence in less fortunate countries, such as Africa, is quite different. In sub-Saharan Africa between 4 and 15% of pregnant women have syphilis, and an estimated 492,000 infants die of congenital syphilis every year worldwide (Schmid 2004).

Despite the great investment of foreign aid in MTCT prevention programmes, this has not been matched by equivalent screening for, and

treatment of, maternal syphilis. This intervention is both simple and cheap. Syphilis in pregnancy causes spontaneous abortion, intra-uterine growth retardation, pre-term delivery, stillbirth and congenital abnormality in up to 50% of cases (Schulz et al. 1987). In Tanzania, 51% of stillbirths, 24% of pre-term live births and 17% of all adverse pregnancy outcomes could be attributed to syphilis. The plea is made that a concerted effort by the relevant agencies could help to avert the tragedy of children escaping HIV infection only to succumb to syphilis (Peeling et al. 2004).

Transmission through Blood Products

The first reports of haemophiliacs presenting with severe immunodeficiency were in 1982, in the USA, when three cases of *P. carinii* pneumonia in patients who had received factor V111 concentrate were identified. In December of that year, an infant with severe immunodeficiency and who had received a blood transfusion was reported. In1985, universal blood and blood product screening was introduced, the causal relationship with HIV having been established. The number of children with haemphilia children who contracted HIV through infected blood products thereafter steadily decreased in the USA (Selik et al. 1993).

1.12.2 - Natural History of HIV Infection in Childhood

In about a third of HIV infected pregnant women, transmission to the infant will occur in the absence of any intervention, yet in most cases the neonate appears normal.

The prognosis for the untreated infected infant is poor, especially in developing countries, where the mortality rate for the first two years of life is 45-69% (Obimbo et al. 2004). In Europe and the USA the comparable rate was 10-20% (European Collaborative Study 1994). In untreated infants (USA), a minority sub-set of about 15-20% was found to have developed AIDS-defining illnesses by the age of 12 months (Blanche et al. 1990).

The introduction of HAART, in 1997, and anti-microbial prophylaxis, where available, has transformed the picture of paediatric HIV infection so that, in the UK, the progression to AIDS has been reduced by 50% and mortality by 80% (Gibb et al. 2003). An association was found with advanced maternal disease and infection in-utero (Ioannidis et al. 2004). A presentation at about three months of age with severe respiratory illness – due either to Pneumocystis pneumonia or cytomegalovirus – is common and carries a high mortality. By 12 months some 70% of infected infants will have become symptomatic and the median age of progression to AIDS is 6 years. A few may not present until early in the second decade. Generally disease progression in untreated children is faster than in adults. In the resource-rich world, HAART, and pneumocystis prophylaxis, have transformed the prognosis for childhood HIV infection, but

elsewhere the situation remains uncertain, though one of the Millenium Development Goals was to halt and reverse the spread of HIV/AIDS.

Diagnosis of HIV Infection in Childhood

Because maternal antibodies acquired through transplacental passage can persist for 18 months, all infants born to HIV positive mothers are themselves antibody positive. Suspected diagnoses in neonates must be confirmed by the demonstration of active infection – either by viral culture or the detection (Yanase et al. 1986) by PCR of viral RNA or DNA (Burgard et al. 1992). Positive results should be repeated at least once, and negative ones checked by serial tests at regular intervals (Husson et al. 1990).

1.12.3 - Immunopathogenesis in Childhood

In general the immuno-pathogenesis of HIV infection is the same in childhood as in the adult, namely, the infection of cells bearing the CD4 surface marker and, in particular, helper lymphocytes and cells of monocyte/macrophage lineage.

The situation in early childhood with respect to the response to HIV infection differs from that in adults in several ways. Firstly circulating blood levels of lymphocytes are higher in the first few years of life – 6-7 x 10^9 /L are normal levels in the early months, in contrast to the adult counts of 1-3 x 10^9 /L. For reference, in adults with HIV infection a lymphopenia (< 800 cells/mm^3) is found in 18%, and in 70% of patients with AIDS (Treacy et al. 1987). 30-70%

of the total lymphocyte count in infants is made up of CD4 cells. The normal CD4:CD8 ratios are 3:1 in the newborn, 2-3:1 in infants aged one to two and 1-2:1 for children over the age of two (Yanase et al. 1986). The high initial viral loads – commonly at $1 \times 10^5 - 1 \times 10^6$ HIV-RNA copies /ml of plasma - do not seem to be associated with HIV-specific CD8 lymphocyte activity, and the marked fall in viral load following initial infection that occurs in adults is not seen. The rate of progression may be slower in the presence of HLA alleles B27 and B57 (Feeney et al. 2004). The immunodynamics of HIV infection in infancy and childhood, especially with regard to the rate of progression, may be related to the degree of involvement of the thymus in HIV infection. There is some evidence that involvement of the thymus can occur soon after HIV transmission to the foetus and, indeed, may contribute to early miscarriage. Abnormalities were found in the thymus of three out of thirty seven foetuses aborted by HIV positive women. These were a degree of lymphocyte depletion and though there was no evidence of any extensive HIV infection it was thought likely that the changes were related to HIV infection (Papiernik et al. 1992). Detailed examination of the thymus of an HIV positive infant, dying at 3 days of age, showed the presence of infection of thymocytes, by in situ hybridisation, and selective depletion of some thymocyte subsets (Rosenzweig et al. 1993).

In a cohort of 59 HIV infected infants, 17 showed depletion of CD4+ and CD8 + T cells suggestive of thymic compromise and 5 infants with the Di George syndrome, who were also part of the study, showed a more rapid

course to AIDS and a higher early death rate than the other HIV positive children (Kourtis et al. 1996).

The other side of the coin is that, if there is no early compromise of thymic function, there is an advantage for children compared with adults in the possession of a relatively larger and more active thymus. This obtains in the apparently greater ability to challenge viral escape mutations with a greater repertoire of cytotoxic T lymphocyte (CTL) responses (Shearer et al. 1997). Another apparent relative benefit is in the regeneration of HIV specific T-helper activity which follows treatment with HAART in children (Rosenberg et al. 2000), but which declines with similar treatment in adults (Lichterfeld et al. 2004)

As regards monitoring, both the plasma viral load and the CD4 count are used, as in adults (Palumbo et al. 1998). However, in view of the declining CD4 counts (absolute CD4 counts are much higher in infancy) which occur normally, a preferable index is the CD4 %, i.e. percentage of total lymphocytes that are of CD4 lineage until the age of five (Waecker et al. 1993). Above this, absolute CD4 counts become as reliable a marker as in adults. In resource-poor settings, clinical examination and simple haematological indices, such as haemoglobin level and total lymphocyte count, may be the only markers available.

Haematology Findings in Childhood HIV Infection

Bone marrow abnormalities are common in children who suffer from HIV infection, but tend to be non-specific and may not necessarily help in the elucidation of haematological problems (Mueller et al. 1996).

Anaemia (10 gm./l or less) is common in childhood HIV infection and a haematocrit of less than 25% is often associated with a poor prognosis (Ellaurie et al. 1990). The anaemia is commonly hypochromic and microcytic and associated with iron deficiency. Rarely is it macrocytic or haemolytic. An association with AZT therapy exists and may be dose-dependent (Englund et al. 1997).

Neutropenia with less than 1,500 white cells / mm^3 has been reported in 43% of previously untreated children with HIV infection (Pizzo et al. 1988) The neutropenia may be isolated, or associated with anaemia and thrombocytopenia. Aetiological factors are bone marrow involvement by HIV or opportunistic infections and, in the case of therapy, AZT.

Thrombocytopenia as an HIV-related phenomenon was noted in 1982, at the start of the epidemic. In a paediatric population a platelet count of less than 50,000 /mm^3 was found in 19% (Butler et al. 1991). The aetiology of thrombocytopenia in childhood HIV infection is not well understood and the response to therapy is variable.

One of the first blood findings in infected infants is B-cell hyperactivity associated with elevation of one or all immunoglobulins (IgG), with IgA and IgM elevated at three months. IgG levels may rise to 1gm/L in the first year and even to 4gm/L later. Despite these levels there is a widespread

abnormality of antibody responses as evidenced by early susceptibility to bacterial infection.

CD8 T-cells, cytotoxic/suppressor lymphocytes, are not depleted until very late in HIV infection. They are considered to play an important role in specific HIV immune response (Borrow et al. 1994), and this is supported by evidence from simian immunodeficiency studies (Schmitz et al. 1999) (Mphatswe et al. 2007).

1.12.4 - Clinical Features – Neonatal Period and Early Infancy

In considering the clinical aspects of HIV infection in children, compared with those of adults, two general points are worth making. The first, which is self evident, is that childhood is a time of somatic and intellectual growth and these attributes are therefore susceptible to interference. The second is that the long asymptomatic period in adults does not have an exact counterpart in childhood for various reasons, not least because childhood is naturally a time of first encounters with common environmental pathogens, but also because significant problems tend to arise before the advent of severe immunosuppression.

The prognosis for the untreated infected infant is poor, especially in developing countries, where the mortality rate for the first two years of life is 45-69% (Obimbo et al. 2004). In Europe and the USA the comparable rate was 10-20% (ECS – 1994). The introduction of HAART in 1997, and anti-microbial prophylaxis where available, has transformed the picture of

paediatric HIV infection so that, in the UK, the progression to AIDS has been reduced by 50% and mortality by 80% (Gibb et al. 2003).

Bimodal Distribution in Early Childhood

In 1988 an analysis of 215 infected infants in New York City suggested two at-risk populations with respect to incubation periods. In the first, designated rapid progressors comprising some 12% (± 7%), a very short incubation period of median 4.1 months was present and for the remainder, the slow progressors, a median of 6.1 years was identified (Auger et al. 1988). The clinical and immunological status of 267 infected neonates (total population born to infected mothers was 1,386) was the subject of a prospective, multicentre trial (Mayaux et al. 1996), which served to identify the markers of susceptibility for the early severe form of HIV in infancy (Blanche et al. 1990) .

Within 2 weeks of birth, using viral culture or HIV PCR, 35% of the cohort was identified as being infected, and was observed to differ from the remainder both in terms of clinical features and laboratory investigations. The infants in this group were twice as likely to have a birth weight below 2500gm, and also had a 1cm. smaller head circumference on average. They were three times more likely to have lymphadenopathy or hepatosplenomegaly. They had a lower mean CD4 count (2.57 vs. 3.49 $x10^9$/L), a higher IgM level and a lower platelet count.

This group comprised 61% of the infants with the early severe form of the disease – that is infants suffering from wasting, encephalopathy and recurrent severe infections occurring within the first year of life. As virus detection by

PCR or culture at birth is considered to indicate pre-existing intra-uterine infection, the implication is that this is one of the main determinants of early disease. The authors pointed out that additional mechanisms must operate to account for the other cases in this group and also that some infants, infected at birth by blood transfusion, also developed early severe disease (Jones et al. 1992) .

The Clinical Course of Rapid Progressors

Non-specific conditions such as hepatosplenomegaly, lymphadenopathy, dermatitis and parotitis are common to both groups, but they occur earlier in the rapid progressors and are more severe.

In the absence of treatment some 15-20% of infected infants will have developed an AIDS-defining illness within one year. Commonly the first illness is a respiratory one at about three months of age and due to Pneumocystis carinii (now jirovecii) pneumonia. This illness occurred in up to 53% of children with AIDS (Rogers et al. 1987) and carried a high mortality (and still does even with treatment) (Williams et al. 2001). Another common respiratory illness is lymphoid interstitial pneumonitis (LIP), or pulmonary lymphoid hyperplasia (PLH), which occurs in about 20-30% of vertically infected children. It is a disorder in which there are diffuse infiltrates of lymphocytes in the lung parenchyma. It can occur in infants as young as 2 months but is more commonly encountered in the one plus age group. The aetiology is uncertain, it may represent an unusual response to Epstein-Barr

virus (Andiman et al. 1985), or an exaggerated response to an inhaled or circulating antigen.

Examination of lungs removed at post-mortem has shown a pleomorphic lymphoid infiltrate of which 46% were B cells, 25% CD4+ T cells and 40% CD8+ T cells (Joshi et al. 1985). The course of the condition is quite variable. It is an AIDS defining condition (CDC classification Group IV E). In some it remains relatively asymptomatic; in others a wheezing condition develops; and in yet others progressive pulmonary disease develops with a bronchiectasis-like syndrome. Treatments tried in addition to standard anti-retroviral therapy are pulmonary toilet with aerosol administration, oxygen and physiotherapy. Its incidence has remained relatively constant at approximately 20% of all patients.

Pulmonary TB is more often a problem in Africa (see section 2.5.3). Gastro-intestinal problems are common and one of the most important, an AIDS-defining condition, is oesophageal candidiasis. (Cello 1988). A degree of hepatosplenomegaly is present in the majority of children with HIV infection.

Bacterial (Bernstein et al. 1985) and viral (Jura et al. 1989) infections are common, frequently severe and were the presenting condition in 20% of infants in one series (Krasinski et al. 1988). The particular susceptibility to infectious disease is considered due in the main part to the B-cell defects associated with CD4 T-cell depletion; although hypergammaglobulinaemia is frequently present, antibodies are generally non-functional.

Cardiovascular disease contributes a small but significant burden to paediatric AIDS sufferers. A study of 433 children with AIDS showed a 6.5%

incidence of cardiomyopathy with a relative risk of dying of 2.8%, compared with other children with AIDS (Tovo et al. 1992).

1.12.5 - The Central Nervous System in Childhood HIV Infection

The Clinical Manifestations of Paediatric HIV Infection

With regard to neuropsychological development, HIV infected children can be conveniently considered in three sub-sets – those with encephalopathy, those with some CNS compromise and those with no apparent deficit of CNS function (Working Group 1991). The first group, that of encephalopathy, can be further sub-classified into subacute progressive, plateau and static. The subacute progressive course is most common in infants and young children – in this there is actual regression of function, where previously acquired skills are lost. In the plateau group new skills are scarcely gained but existing ones are retained, and in the static group there is a continued delay in progress. In the more severe cases of encephalopathy there may be moderate to severe psychomotor difficulties.

The HIV-related compromise group fall within the normal range but there is impairment of some neurocognitive functions.

The apparently normal function group do not differ from their fellows in any specific way or show decline over time.

A degree of correlation between cognitive function and HIV disease state was noted in the pre-treatment era, using laboratory markers such as CD4 counts and p24 antigen levels (Brouwers et al. 1994).

Almost half (Mason et al. 1990) of the rapidly- progressing infant sub-set will acquire an encephalopathy, within three years, characterized by hypertonic diplegia and developmental delay – especially in respect of motor skills and language (Lobato et al. 1995). Dementia may supervene. CT imaging may show a degree of atrophy and calcification in the basal ganglia. The median survival from onset is 11 months (Scott et al. 1989) . The very high mortality rates quoted above – 32% in first year and 52% in the second – mean that many will die before the possible expression.

In children with a later onset of AIDS at a median age of 6.1 years (Scott et al. 1989), the spectrum of disease is similar to that outlined above.

A general phenomenon in HIV infection in children is disturbance of growth, which is manifest at all ages. Inextricably linked to the problem of growth and development in HIV-infected children, certainly in the developing world, is malnutrition. This condition – specifically protein calorie malnutrition (PCM) – is the most common cause of immunodeficiency worldwide. Indeed, before HIV infection was recognised, Kaposi's sarcoma and *Pneumocystsis carinii* pneumonia were described in malnourished, but otherwise healthy, children and adults, in resource-poor settings (Hughes et al. 1974).

The Neuropathology of Paediatric HIV Infection

Much of what is known of the neuropathology of childhood HIV infection is derived from the American experience of the epidemic. In some of the literature the ethnic background of the study population is detailed, but often it is not. The incidence of HIV infection in America has been unevenly

represented in the population sub-sets – in the general population the main racial groups are distributed thus: 56% white, 20% black and 21% Hispanic. Of the 7629 children in America reported to CDC by December 1996, 58% were black, 23% were Hispanic and 18% were white (Centers for Disease Control and Prevention 1996). Genetic influences may play a part in determining the susceptibility to, severity of, and rate of progression of HIV related CNS pathology. Another point in considering the results of studies is whether or not there could be a bias in favour of one or other arms of the bimodal distribution (Auger et al. 1988) which clearly existed before treatment was available to modify transmission.

a. Cerebrovascular disease: this is a cause of morbidity and mortality in childhood HIV infection. Arteries affected by an inflammatory process were seen in the brains of children with HIV encephalopathy (Joshi et al. 1990). Of seven children with HIV infection who developed strokes, six of them were found at autopsy to have a thrombotic cause associated with vascular disease, and two showed aneurysmal dilatation of the circle of Willis (Park et al. 1990). The pathogenesis of vessel pathology in HIV infection is uncertain. Direct infection of the endothelium, though detected by some investigators, has not been a universal finding (Lipshultz et al. 1990). Circulating cytokines have also been postulated as an aetiological factor (Weiss et al. 1993)

b. CNS opportunistic infections: as noted above, children are generally less liable to opportunistic infections than adults and this was reflected in the relatively low incidence (11 out of 114 cases) in a multi-centre post mortem study of children with AIDS (Kozlowski 1990).

CNS lymphoma in childhood is uncommon (Epstein et al. 1988).

The HIV specific abnormalities once frequently observed in the CNS are now seen much less commonly. HIVE was seen in over 50% of infected children in the early days (Belman et al. 1988) but became uncommon in the USA and Europe a few years later (Tardieu et al. 1995).

c. Microcephaly: before the introduction of antiretroviral therapy, this was the most frequent abnormality in paediatric AIDS, seen in 63% of necropsies (Kozlowski et al. 1997). Microcephaly refers to a small head size for the age of the individual and implies small brain volume. In effect this phenomenon was equivalent to growth retardation. A detailed study of 12 brains from children suffering from AIDS and 7 control brains was performed in 1997(Kozlowski et al. 1997). The AIDS brains showed significant differences in volume when compared with both the control brains and the standard reference values for normal children of similar ages. The reduced volumes were shown to affect both the total of the hemispheres and of the individual structures, including separately the whole of the cortex, the white matter and the basal ganglia. The negative weight differences for the AIDS brains ranged from 13.2-55.2% when compared with normal brains of the same age. Cerebral atrophy had also been demonstrated by CT scanning, and in a systematic unbiased study (DeCarli et al. 1993) of children with symptomatic HIV disease in 1993, ventricular enlargement and widening of sulci were seen in 78% of cases, and attenuation of the white matter in 27%.

d. Cerebral vascular calcification: this is a common finding in childhood HIV infection, especially in the basal ganglia. It is not seen in adults (Kure et

al. 1991) and tends to be a feature of vertically infected children. In a CT scan study it appeared more than twice as common in vertically infected children compared with transfusion infected ones, 36% vs. 14% (Brouwers et al. 1994). Furthermore as calcification was rarely seen in premature infants infected by transfusion after 34 weeks, the implication was that it might indicate intrauterine infection and, possibly, local breakdown of the blood brain barrier (Belman et al. 1986; Kinney et al. 1988).

e. Inflammatory cell infiltrates: both perivascular cuffs and parenchymal collections of macrophages, with giant cells, were common, especially in infants with progressive encephalopathy, signifying a high prevalence of HIVE in Western paediatric populations (Dickson et al. 1989). These changes were found mostly, but not exclusively, in the deep white matter and basal ganglia.

f. Neuronal loss and apoptosis: this was reported to occur in the younger age group with encephalopathy (Wiley et al. 1991) but was not confirmed in other studies (Brouwers et al 1994).

g. White matter pallor with astrocytosis: this was found in most paediatric AIDS cases in the pre-treatment era, on staining brain sections with Luxol fast blue (Epstein et al. 1986) (Kozlowski et al. 1997). It may be hard to interpret myelin pallor in the first two years of life, when myelination is still active, especially if there is delay due to adverse conditions, (Kinney et al. 1988). Delay, or arrest, of myelination may be a major component of the substrate of encephalopathy. The spinal cord may also show delay in myelin maturation but in some AIDS cases, axonal injury co-exists (Dickson et al. 1989).

1.12.6 - Treatment of HIV/AIDS in Children – in the developed and developing World

In general the approaches to treatment of HIV infection in childhood are broadly similar to those used in adults. The three chief determinants are the presence of symptoms, the CD4+ T cell count and the viral load.

Treatment should be started in symptomatic children regardless of CD4+ T cell count or viral load and, conversely, since the CD4+ T count and/or the viral load are predictive of disease progression and death, treatment may be indicated even in the absence of symptoms. The guidelines vary slightly between Europe and the USA and according to age. For children under a year, values which should prompt treatment are a CD4+ count of 20% or less and a viral load of HIV-RNA 1,000,000 copies/ml of plasma or greater; for children over a year, a similar CD4+ T cell count or a viral load of greater than HIV -RNA 100,000 copies/ml of plasma.

A combination of two reverse-transcriptase inhibitors and a protease inhibitor is considered optimal therapy (MMWR – 1998). Therapy can suppress viral loads to undetectable levels and restore CD4 counts (Fraaij et al. 2005). The problems of drug resistance and side effects encountered in the treatment of adults also occur in childhood, and additional difficulties are those of appropriate formulations and volumes, coupled with an often relatively larger dosage requirement.

Continuous monitoring is required at three monthly intervals and unfavourable indices, or symptoms suggesting intolerance, indicate the need

for a review of therapy. Since HAART became an integral part of the management of HIV infected children in the developed world, most affected individuals survive into adulthood.

In the developing world, treatment programmes are in the early stages of implementation, and the G8 target of universal availability is set for 2010. Initial studies, based on a start date of 2004 and investigating children in Côte d'Ivoire, Kenya and South Africa, have shown promising results (Rouet et al. 2006), (Nyandiko et al. 2006) and (Reddi et al. 2007).

1.12.7 -The Future for Paediatric HIV/AIDS

The great advances in prevention of mother-to-child-transmission and treatment with HAART, achieved in the developed world, provide a yardstick against which progress in poorer countries can be measured. The aims must be wider access to testing for pregnant women, with pre- and post- confinement intervention as necessary. Likewise early diagnosis in the neonate with appropriate treatment should be a goal. There is increasing goodwill among the young people of the richer nations, "Live-AID" for example, and the hope is that, with the provision of adequate finance and encouragement, poorer countries will able to start reversing the burden of paediatric HIV infection and AIDS.

1.12.8 - Differences between HIV Infection in Adults and Children

An absolute difference is in the integral nature of somatic and intellectual growth in childhood, and the possibility of its impairment. Also related to the development of the child are the first-time encounters with environmental pathogens and the associated increased risk of meeting these with impaired immunity. For example, the association of LIP with Epstein-Barr infection. This of course means that the illnesses seen in adult AIDS as a result of reactivation of past and previously quiescent disease, for example, herpes simplex, CMV, and toxoplasma (cerebral or other site), generally have no counterpart in the spectrum of paediatric AIDS. Primary lymphoma of the brain occurs infrequently (Epstein et al. 1988). Malignancy is uncommon in childhood and accounts for less than 2% of AIDS-defining illness. Kaposi's sarcoma is extremely rare (Joshi et al. 1990). The fairly clear-cut bimodal disease distribution of childhood does not occur in adult HIV infection.

Other differences emerge in laboratory investigations. For example, anaemia in childhood HIV infection is commonly hypochromic and microcytic, whereas anaemia in adult HIV infection is typically normochromic and normocytic. Lymphocyte counts are physiologically higher in the first few years of childhood and the hypergammaglobulinaemia which is common in children is infrequent in adults. The initial viral load is higher in children.

1.13 - What is known of Paediatric African AIDS?

HIV Infection in Africa

The HIV/AIDS epidemic in Africa differs from that in Europe and North America in several ways. It is mainly transmitted heterosexually, with a male to female ratio of 1:1 and the homosexual and drug abusing risk groups are probably of negligible proportions. The prevalence rate for adults in sub-Saharan Africa is much higher. All of the 14 countries estimated by WHO to have rates of infection greater than 5% are in sub-Saharan Africa. The overall rate in Africa was 8.8% in 2000, of which 55% was female. Infection may be facilitated by a higher incidence of co-existing sexually transmitted disease, eg syphilis, and by different sexual customs, for example, intercourse at a younger age for women.

In women attending ante-natal clinics, in East and South Africa, rates of between 14-33% have been recorded. The mother-to-child transmission rate without intervention is higher and ranges from the previous (untreated) rate of 15-30% in Europe to 25-40% in sub-Saharan Africa (Nesheim et al. 1994). Transmission by contaminated blood or needles may still be a problem in some parts of sub-Saharan Africa. The progression to AIDS may be more rapid in the presence of other tropical diseases – for example malaria and helminth infection.

The Clinical Course in African Children

The high prevalence of HIV/AIDS for adults in sub-Saharan Africa has a significant impact on the rate of vertical transmission. In some parts of Africa the proportion of women of childbearing age who are HIV positive reaches 35% or more (Newell et al. 2004). This is reflected in the high incidence of paediatric HIV infection (18.3% for children in a hospital setting) (Blantyre) (Molyneux et al. 2003). In this study the commonest causes for admission were gastroenteritis, pneumonia, TB and malaria. In cases of malnutrition, 62% were HIV positive and in cases of probable TB a rate of 58% was recorded. The aim of the study was to compare the effect of HIV infection on the management of bacterial meningitis, which had previously been shown to account for 2.7% of all admissions (Molyneux et al. 2000). Out of 598 cases of meningitis 459 were tested for HIV status and 157 (34%) were found to be HIV positive. The overall mortality rate was 65% in the HIV positive group and 36% in the HIV negative group.

The data shown above serve to illustrate the spectrum of illness in sub-Saharan Africa. Without treatment the mortality rate for HIV infected children at two years is estimated to be 45-59% (Dabis et al. 2001), against that in Europe and the USA before treatment of 10-20% (European Collaborative Study 1994). In one study of perinatally HIV infected children in Central Africa, 89% had died by the age of 3 years (Taha et al. 2000).

Limited resources, in even simple matters such as access to clean drinking water, mean a higher background level of gastro intestinal disease. Social deprivation imposed by orphan status and malnutrition compound the desperate

situation for even HIV negative infants; the mortality risk for HIV positive children is ten times greater (Newell et al. 2004).

The bimodal expression of HIV disease is similar in African populations but disease progression in both sub-sets appears more rapid. Diarrhoea is the commonest cause of death and rotavirus the usual pathogen. The risk of death in chronic diarrhoea is eleven times greater for HIV positive infants (Thea et al. 1993).

Respiratory Illness

Pulmonary infectious disease is a particularly important cause of morbidity and mortality in African children. Reference is made to this in the original Abidjan study, and a further post-mortem study, conducted in Zambia and specifically applied to lung disease, is particularly instructive (Chintu et al. 2002). In this study of 264 children, aged between 1 month and under 16 years, 137 were male (93 HIV- 1 positive and 44 HIV-1 negative) and 127 were female (87 HIV-1 positive and 40 HIV-1); examination was confined to the lungs.

Multiple pulmonary diseases were common, but overall the four main conditions were: acute pyogenic pneumonia – 39.1%, *Pneumocystis carinii* pneumonia – 27.5%, cytomegalovirus – 20.2% and tuberculosis – 18%. In the HIV-1 positive group the three commonest conditions were: acute pyogenic pneumonia (41%), *P.carinii* pneumonia (29%) and CMV (22%) and for the HIV-1 negative group, acute pyogenic pneumonia (50%), tuberculosis (26%) and interstitial pneumonia (18%). Of the 10 cases in which LIP was detected, 9

were HIV-1 positive. TB was common in all age groups, even in the very young, and irrespective of HIV-1 status. It was found as the sole pathology in 25 cases.

The high incidence of TB is a matter of great concern, and its intimate relationship (worldwide, as well as in Africa) with HIV is a formidable partnership from a public health point of view (Chintu et al. 2002). The percentage of HIV positive children in South Africa co-infected with TB approaches 50% (Jeena et al. 2002). HIV is estimated to increase the risk of contracting pulmonary TB by up to 20-fold (Madhi et al. 2000). Diagnosis is difficult and treatment complicated by differing regimes, side effects and the emergence of resistant strains.

Malaria

The relationship of HIV infection with malaria, once thought to be a mutually neutral one, is now considered to be disadvantageous with respect to the incidence and severity of malaria and the HIV viral load.

Plasmodium falciparum is the species most likely to cause severe disease, with cerebral complications. There are reckoned to be 515 million cases of malaria in the world each year, of which over 70% occur in Africa and mostly affect children. Infants are less susceptible to malaria in the first six months, despite being infected, because of maternal antibodies. Seizures are a common presenting feature of cerebral malaria. Severe anaemia, hypoglycaemia and coma are other manifestations. A longitudinal study in 1991 found that children with AIDS had an increased incidence of malaria and higher parasite

densities than pre-AIDS or non-infected children (Greenberg et al. 1991). Another study showed that HIV-1 infection was associated with an increase in severe malaria with coma and the prospect of neurological sequelae (Grimwade et al. 2003).

A prospective study in adults showed that infection of HIV-1 positive individuals with P.falciparum malaria led to significant increases in HIV-1 RNA, which was thought possible both to accelerate disease progression and facilitate transmission (Kublin et al. 2005). A prospective trial of cotrimoxazole prophylaxis, anti-retroviral therapy and insecticide-impregnated bed nets found a significant reduction in malarial episodes in HIV-1 positive adults (Mermin et al. 2006).

Mathematical modelling on a Kenyan population suggests that recurrent infection with malaria in HIV-1 positive individuals is a significant factor in enhancing transmission (Abu-Raddad et al. 2006). Perhaps it should be added that it is still thought unlikely HIV-1 transmission could occur by mosquito bites.

1.14 - Background to the Present Study

The present investigation arises from a study undertaken in 1995-6 in Abidjan, Ivory Coast, by Lucas and Bell (Lucas 1996, Bell 1997) which was based on a series of children coming to autopsy and designed to reveal the contribution of HIV infection to causes of death as compared with local mortality in HIV negative children. Virtually no clinical information was

available for these children who were moribund or dead on arrival at the hospital. Conclusions as to cause of death were based on post mortem HIV serology and autopsy findings. Consequently CD4 counts were unavailable. This study was the first to document accurate pathology findings in an African population in contrast to other studies that have inferred pathology from clinical data alone (Valdez and Lederman 1997). Furthermore, the very difficult conditions under which the necropsies were conducted should not be underestimated.

Of the 408 children arriving in the mortuary during the study period, 80 (20%) were found to be HIV positive (two of them HIV-2 rather than HIV-1 positive). Children aged one month or older, and up to 12 years, often selected on size since age was not actually known, were included in the study after the HIV status was determined. 78 HIV positive and 77 HIV negative children were studied in full autopsies. Infective illnesses were the cause of death in almost all HIV negative and positive children, as determined at post mortem examination. The spectrum of disease was similar, except that certain conditions, such as *Pneumocystis carinii* pneumonia (8 cases all under one year), lymphocytic interstitial pneumonitis (1 case), cerebral toxoplasmosis (3 cases) and multinucleate giant cell encephalitis (2 cases), were seen only in HIV positive children. AIDS-defining conditions were found in 24 of the 36 (67%) children under the age of 15 months, and 12 of the remaining 42 (29%).

Respiratory tract diseases were the dominant causes of death in all children underlining the importance of respiratory illness in the inpatient paediatric population, HIV positive and negative, in Abidjan (Vetter et al. 1996).

Measles was the cause of death in thirteen HIV positive children and five HIV negative children. Miliary tuberculosis was seen only in one 18-month-old HIV positive child and two HIV negative children of 36 months and 8 years. Enteric illness children was listed as the cause in six HIv positive and four HIV negative children. Purulent meningitis accounted for the deaths of eleven HIV positive children and nine HIV negative children. Apart from the cases of HIVE and was listed as the cause of death in six HIV positive and four HIV negative toxoplasmosis mentioned above, two other cases of encephalitis were seen, one due to measles and the other to cytomegalovirus. Malaria was present in six HIV positive and twenty-three HIV negative children, and was thought to be the cause of death in three and eighteen respectively.

In discussion of the systemic pathology the authors commented on several key findings. The prevalence of pneumocystis pneumonia, at 31% in HIV positive children, contrasted strongly with the rate of 3% in HIV positive adults at post-mortem in Abidjan (Lucas et al. 1993) , and yet was comparable to that found in Europe and North America. Though measles was common, HIVE and tuberculosis were not, probably reflecting a rapid progression to death in these vulnerable children.

The neuropathology findings in this paediatric cohort were described in 1997 (Bell et al. 1997). The cases were divided into those aged one to fourteen months and those older than fifteen months. Comparison of brain weights between HIV positive and negative children showed the mean weights for both younger and older HIV positive groups to be less than their HIV negative counterparts. For those under fifteen months the mean weights were 786 gm.

for HIV positive, and 940 gm. for HIV negative cases ($p = < 0.005$). For those aged fifteen months and over, the respective weights were 1,119 gm. and 1, 211 gm. ($p = <0.001$). However ventricular dilatation was not a significant feature of HIV positive cases and cerebral atrophy was not noted. Florid meningitis was visible in twenty brains, one of which proved on microscopy to be tuberculous, in an HIV negative child. Eight brains showed haemorrhages in association with malaria. One tumour was found in the cerebellum of an HIV negative three year old and was later shown to be a medulloblastoma. The brains were sampled comprehensively for histology. All brain sections were examined with routine stains and in HIV-1 positive children were screened for HIV-1 p24 antigen. Immunocytochemistry was used only in very selected cases for glial fibrillary acidic protein (GFAP); and for specific cell markers for microglia or macrophages (CD68) and lymphocyte subsets - CD45 (leucocyte common antigen LCA), CD3 (a marker for CD4+ and CD8+ lymphocytes), MT1 (which stains the surface membranes of all T cells, immature B cells and histiocytes) and L26 (a marker for CD20 lymphocytes).

HIVE was found in the brains of four children. Two of these cases, one aged five and a half years and the other six years, had HIV p24 positive giant cells in their white matter. The other two, both aged four months, showed foci of HIV p24 positive microglial cells without giant cells. Leucoencephalopathy was present in three out of the four, and in another brain affected by CMV encephalitis. Toxoplasmosis was present in three brains (including one of the two with HIV encephalitis). Severe necrotizing encephalitis in a five year old was confirmed as due to measles by immunohistochemistry. Other conditions

present in both HIV positive and HIV negative children included micronodular encephalitis which was found more often in HIV positive cases than in HIV negative cases. Basal ganglia mineralization was more pronounced in the HIV positive group, especially in younger children. Non-specific inflammatory cell infiltrates were of variable degree in both groups. White matter pallor and gliosis was twice as common (26 cases) in HIV positive children compared with HIV negative children (13 cases). Acute bacterial meningitis was equally represented in both groups but malaria was more common in HIV negative children (11 cases) than in positive cases (n=2). One three-year old HIV negative child was thought to have trypanosomiasis. Another feature common to both HIV positive and negative children was the presence of meningeal and perivascular lymphocytic infiltrates, found in 26 HIV positive but in only 11 HIV negative children. It is of note that low grade lymphocytic infiltrates in the CNS are a typical feature of presymptomatic HIV infection and it is important to note their occurrence in HIV negative children in this series. A pleomorphic lymphocytic infiltrate was present in two other HIV negative children and shown by immunohistochemistry to be due to Epstein-Barr virus. In Africa this infection is commonly acquired in the first year of life, as opposed to throughout childhood in the developed world. Of the two HIV-2 positive children, the younger child, aged two months, showed micronodular encephalitis of indeterminate cause and the seven year old showed an apparently normal brain.

When correlating the neuropathology findings with the systemic pathology several pertinent points were noted. The four cases of HIVE occurred

independently of AIDS-defining illness elsewhere in the body, the cause of death being pyogenic pneumonia in all. *Pneumocystis carinii* pneumonia was the cause of death in both cases of CMV encephalitis and in the two children with the Epstein Barr positive meniongeal infiltrate. The causes of death in the twenty-four HIV positive children with low-grade lymphocytic meningitis were measles-associated pneumonia in seven, and bacterial pneumonia in the remainder.

In discussing these findings, the authors stressed that the presence of CNS abnormalities in 64% of HIV negative children formed a standard against which findings in HIV positive African children could be assessed. The naturally high infant mortality in Africa was considered a possible reason why the bimodal distribution of HIV infection, observed in American children, was not displayed in the population studied in Abidjan. The spectrum of disease, however, was not dissimilar if allowance was made for the superimposition of tropical disease. In particular, bacterial disease was common to both locations. However a major difference between the African and American cohorts was in the prevalence of HIVE – 6% in the Abidjan paediatric population and 38% in the US series (Kozlowski 1990). The higher infant mortality in Africa and a possible autopsy selection bias in America were thought possible contributors to this discrepancy. However, it is of interest that the rate of HIVE encephalitis in Abidjan adults was also low (3%) (Lucas et al. 1993). The finding of lymphocytic meningitis in 34% of HIV positive children was potentially a marker of pre-AIDS, as in adults (Bell et al. 1993), but the similar

finding in HIV negative children was considered to diminish the potential usefulness of this approach.

The comparable incidence of opportunistic infections between the African and American populations was noted, though toxoplasma had not been found in American paediatric AIDS series. However there is a high environmental incidence of this parasite in Africa, and cerebral toxoplasmosis was found in about 15% of adults dying of AIDS in West Africa (Lucas et al. 1993). Cytomegalovirus (CMV), found systemically in 3.1% of HIV positive children in the series, was also detectable by serology in 77% of mothers in Abidjan (Lucas et al. 1993). Tuberculosis, in contrast to its low expression in the HIV-positive children in the series, was detected in 11% of adult in-patients with AIDS in Abidjan (Lucas et al. 1993) although later studies found the prevalence to be even higher (Chintu et al. 2002). HIV infection did not appear to influence the likelihood of developing acute bacterial meningitis. In contrast, malaria appeared to be more common in HIV-negative children. The reduced brain weight seen in HIV infected children in America is also seen in those in the West African study. Various mechanisms have been invoked in the pathogenesis of impaired brain growth, including neuronal apoptosis (Gelbard et al. 1995), (Adle-Biassette et al. 1995), defective methylation (Surtees et al. 1990), astrocytic infection (Saito et al. 1994) and cytokine expression (Elovaara 1995).

1.15 - The Present Study: Hypotheses and Aims

The previous studies of the African paediatric cohort were limited to basic neuropathological descriptions with no detailed investigation of cellular reactions in the brain. Given the high level of pathology in the brains of the HIV negative children, careful comparison with HIV positive brains is clearly vital, but it is accepted that study of these cases cannot reflect the normal state of African paediatric brains and conclusions should be suitably cautious. So little is known of African neuropathology, and paediatric cases in particular, that this cohort warranted further investigation, at the very least with a wider immunohistochemical panel to investigate CNS cellular sub-sets. Although the affinity of HIV for microglia and also for astrocytes is well known, and both cell types display an activated phenotype in the HIV infected brain, the contribution to cell populations of hyperplasia, hypertrophy, increased cell entry (in the case of monocytes becoming microglia), cell division and apoptosis is not well understood. Variations in the Apolipoprotein E (APOE) genotype are known to influence neuroimmune mechanisms and response to CNS injury, and has been investigated in the context of HIV infection in Caucasians but never in African populations.

Accordingly, the following **hypotheses** were formulated:

1. That there would be significant differences in the glial cell numbers between HIV positive and negative children, specifically a likely increase in the numbers of microglia and astocytes in the former group.

2. That the HIV positive children would show a higher level of activated microglia and infiltrating lymphocytes than shown by the HIV negative children, despite the high level of background brain pathology.
3. That the degree of inflammatory response would vary according to the individual APOE genotype, with likely maximum response in individuals carrying APOE ε4 alleles.

In order to address these hypotheses, the present study is presented in three parts, outlined in Chapters 3, 4 and 5.

In the **Initial Study (Chapter 3),** a quantitative assessment was made of different cell sub-sets in selected areas of the white matter in age matched HIV positive and negative children.

In the **Main Study (Chapter 4),** microglia/macrophages (innate neuroimmune system) and lymphocytes (adaptive neuroimmune response) were quantified and compared in selected HIV positive and negative children.

In the **APOE Study (Chapter 5),** APOE genotype was determined and related to the degree of microglial activation in each case.

Chapter 2: General Materials and Methods

2.1 - Source of Cases

The material for this study was obtained from a comprehensive post-mortem survey of children who died in the community, or in the largest hospital, in the capital city of the West African country of Côte d'Ivoire, Abidjan. This survey was undertaken between August 1991 and May 1992 by Sebastian Lucas, Professor of Histopathology, Guy's and Thomas's Medical School, London, with the aim of documenting the range of HIV and associated disease in African children. Jeanne Bell, Neuropathologist at the Department of Pathology, University Medical School, Edinburgh, completed the neuropathological examinations in this cohort.

The selection of cases was as follows – all children brought to the mortuary were serologically tested for HIV, and consecutive necropsies were performed on all those found to be, or suspected of being, HIV positive. Post-mortems were also performed on a randomly selected cohort of HIV negative children, matched for age and height. The age of the children was not always available and for these an estimate was made from measurement of crown-heel length and comparison with a previously constructed chart of height and age. Children aged one month or older were included in the study – a length of 56 cm or less was taken to indicate an age of less than one month (Schulz et al. 1962). The upper age and height limit was 12 years and 140 cm. As regards

the antecedents to death, very little clinical, laboratory or radiological information was available.

HIV testing was performed on autopsy blood samples using mixed antigen assay for HIV-1 and HIV-2, enzyme-linked immunosorbent assay (ELISA) and Western blotting. For children under the age of 15 months the influence of maternal antibodies was discounted by Western blotting for HIV-IgA – in all for 51 children nominally under the age of two.

The study population was composed of 78 HIV positive and 77 HIV negative children with median ages of 18 months (range 3 months – 8 years) and 21 months (range 3 months – 12 years) respectively. The total number was 155, of whom 78 were male and 77 were female (Table 2.1.1).

Table 2.1.1 - The Abidjan Paediatric Cohort

Age in months	HIV +ve cases	HIV –ve cases
1-14	36	29
≥ 15	42	48
Totals	78	77

Of the 78 HIV positive children, two were HIV-2 positive. Of the 36 children aged 1-14 months and apparently HIV positive, eight proved to be of indeterminate status by HIV IgA Western blotting.

Complete necropsies, apart from removal of the spinal cords, were performed within 24 hrs of death. The brains were fixed in formalin for two to three

weeks before weighing and dissection. The causes of death and general post-mortem findings have been published (Lucas et al. 1996) and the general neuropathology findings have been studied (Bell et al 1997). The naked eye appearances of the brains were recorded prior to sampling from the frontal, parietal, occipital and temporal lobes, the thalamus, the basal ganglia and the cerebellum – all on both sides - and the mid-brain, pons and medulla.

The present study is based on more detailed examination of some of the blocks of brain tissue from this cohort, now held at the Wilkie Building, University of Edinburgh. **The study was undertaken in three parts**. For the histological studies four regions of the CNS were chosen for study – the cerebral cortex, the hippocampus, the basal ganglia and the cerebellum.

The **Initial Study (**Chapter 3**)** was directed to the white matter and to older cases where myelination was likely to be well underway. Accordingly the blocks were selected to include areas of the brain with well-defined longitudinal fibre orientation, such as the anterior commissure or the internal capsule. The plan was to sample white matter within the chosen section where the preferred orientation of fibres was in longitudinal, i.e. linear or curvilinear, array. However despite careful examination of previously stained sections from the original study, and the blocks themselves, it was apparent that this was not possible for all cases because of the variation of original sampling at post-mortem or that the requisite blocks were not available. In the case of the internal capsule the oblique cut of fibres dictated a departure from this plan but contrary to expectations satisfactory results were obtained. In the case of the cortical sections, because of varying availability, frontal or parietal cortex was

used. Initial examination was of the original haematoxylin and eosin (H&E) sections, or fresh sections cut at 5μm and stained in the same way, and also with Luxol fast blue (LFB). Immunohistochemistry for glial fibrillary acidic protein (GFAP) was undertaken and case selection is detailed in Chapter 3.

The **Main Study** (Chapter 4**)** focused on inflammatory changes in both grey and white matter, using a range of special stains and antibodies. Cases were selected from a wider age range in the cohort (details in Chapter 4) as compared with the Initial study and blocks from the basal ganglia and hippocampus were examined. Details of histological and immunohistochemical methods are detailed below.

In addition, a study was undertaken of APOE genotyping, the **APOE Study** (Chapter 5). For this study the cases used in the Main Study were selected together with a small extra sub-set from the original cohort. Case selection is detailed in Chapter 5. Methods for DNA and APOE genotyping are provided below.

2.2 - Immunohistochemistry

Immunohistochemistry was undertaken using the antibodies shown in Table 2.2.1. and either the Avidin-Biotin Complex (ABC) or Tyramide Signal Amplification (TSA) protocols, copies of which are filed in the appendix.

Sections were counterstained with haematoxylin.

Table.2.2.1 – Antibodies and protocols used in the present study

Stain	Tissue target	Manufacturer	Pre-treatment	Dilution	Method
CD68	Microglia / Macrophages	Dako	Citric acid microwave (mw)	1/150	Avidin-Biotin Complex (ABC)
HLA-DR	Microglia/ Macrophages	Dako	Citric acid m/w	1/100	ABC
CD14	Perivascular Macrophages in CNS	Novocastra	EDTA m/w	1/50	Tyramide signal amplification (TSA)
CD16	Activated Macrophages	Novocastra	EDTA pressure cooker	1/35	TSA
CD8	CD8 T-cells	Novocastra	EDTA Pressure cooker	1/50	ABC
CD20	B-Cells	Dako	Citric acid m/w	1/300	ABC
GFAP	Astrocytes(Most)	Sertotec	None	1/1,500	Polyclonal ABC
βAPP	Axonal Damage	Chemicon	Citric acid/formic acid m/w	1/100	ABC
MBP	Oligodendrocytes/ Myelin	Dako	Citric acid m/w	1/500	Polyclonal ABC
Transferr -in	Oligodendrocytes.	Dako	None	1/200	Polyclonal ABC
HIV p24	HIV-1 Infected Cells.	Novocastra	Citric acid	1/200	Tyramide Signal Amplification (TSA)
Ki-67	Cells In Proliferation	Dako	EDTA m/w	1/50	Polyclonal ABC
Von Willebra-nd factor	Endothelial Cells	Dako	Citric acid m/w	1/400	TSA

Key to Table 2.2.1: EDTA is ethylene-diaminetetracetic acid – a chelating agent. Details of methods for Avidin-Biotin-Complex (ABC) and Tyramide Signal Amplification (TSA) are included in the Appendix..

Notes on Individual Immunostains

CD68 is a highly glycosylated membrane protein expressed strongly in the cytoplasm of cells of monocyte/macrophage lineage, and whilst the antibody detects activated microglia readily, resting microglia are less susceptible (Falini et al. 1993).

The human leucocyte antigen (HLA) system was discovered as a result of a transfusion reaction. The main role of the HLA-DP, DQ and DR molecules is to present antigenic peptides to CD4+ T cells and they are expressed on antigen presenting cells such as B-lymphocytes, monocytes and dendritic cells. In the context of neuropathology, the antibody is used as a marker for perivascular macrophages and activated microglia (Vinci et al. 1984).

CD14 – the CD 14 molecule has a molecular weight of 55kD and is expressed on certain cells of monocyte/macrophage lineage. It has been proposed as an exclusive marker of perivascular macrophages in the brain (Grimm, MC – 1966).

CD16 – the CD16 molecule has a molecular weight of 50-70 kD and is expressed on natural killer cells, granulocytes and activated macrophages (Venneker, GT – 1994).

The CD8 molecule is a polypeptide composed of two chains and has a molecular weight of 32kD. It is found on a T-cell subset of normal cytotoxic/suppressor cells and serves as a receptor for class 1 MHC molecules (Ledbetter et al. 1981).

The CD20 molecule is a transmembrane non-glycosylated protein expressed on B-cells and B-cell precursors but is lost on their transformation to plasma cells. T-cells are not labelled (Mason et al. 1990).

Glial fibrillary acidic protein (GFAP) is a 52 kD intermediate filament found almost exclusively in glial cells or cells of glial origin (e.g. astrocytes, ependymal cells and Schwann cells). There is no cross reactivity of GFAP antibody with vimentin, neurofilaments, cytokeratins or desmin (Eng et al. 1971).

β Amyloid precursor protein (βAPP) is widely expressed in the brain and body. It is also detectable in axons as a marker of early injury in brain trauma or infective disease, moving to the site(s) of axonal injury by fast axonal transport and accumulating where the cytoskeleton is disrupted (Sherriff et al. 1994).

Myelin basic protein (MBP) is produced by oligodendrocytes, is located in myelin and accounts for 30% of the total myelin protein. The staining pattern is similar to that obtained with Luxol fast blue (Hardy et al. 1996).

Transferrin is a plasma protein involved in iron metabolism. In the brain it is stored selectively in oligodendrocytes (Gerber and Connor 1989).

HIV p24 – a marker of HIV infected cells – p24 is a nucleocapsid protein (Kaluza et al. 1992).

Ki 67 – is a cell proliferation marker – the Ki-67 antigen is expressed during all active phases of the cell cycle but is absent in resting cells (Cattoretti et al. 1992).

Von Willebrand factor – is composed of a series of macro molecules which are synthesised in capillary endothelial cells and megakarycytes, and which are essential for efficient blood clotting. The first step of this is adhesion of platelets to the vessel wall. Von Willebrand disease is a genetic disorder transmitted in autosomally dominant manner, and in which there is deficiency of one or more components of the factor. In immunocytochemistry it is useful as an endothelial cell marker (Sehested and Hou-Jensen 1981).

Note: CD4 immunostaining was **not** attempted in this study as it has been found to produce sub-optimal results in paraffin-wax preserved tissue (McCrossan et al. 2006).

2.3 - Microscopy and Quantitation

The white matter was examined using the following stains: haematoxylin and eosin, Luxol fast blue (LFB) and immunostains. For LFB, the sections were laid out in case number sets, in random order, on a light box (X-ray viewing box) and scanned at a distance to judge the overall degree of staining and to compare in a very approximate way the difference between cases. Sections were then examined under the light microscope, first under the x4 objective lens to assess the general appearance of the tissue and then, under the higher power lenses, to note detail of any diffuse or focal abnormalities. The results were then tabulated.

Immunostained sections were examined under the light microscope and the presence or absence of positivity, or pattern of staining, was recorded. Areas of white matter were selected in the cortical blocks in the short arcuate fibres (CNS region 1), in the core of one or more gyri (CNS region 1a), and in the core of the parahippocampal gyrus (CNS region 2), as well as in the fasciculi of the internal capsule (CNS region 3) and at the bases of the folia in the cerebellum (CNS region 4). For the purposes of the Initial study in which cell counts were undertaken, image analysis software, Image-Pro 4.5 supplied by MediaCybernetics and coupled to an Olympus BX40 microscope, was used to capture images, using the x40 objective lens. At this magnification the single frame dimensions were: width = 164.18μ, height = 123.29μ and area = 20,243.8 sq.μ. Sections were examined blinded to age, sex and HIV status.

More detail is provided in the Initial Study, Chapter 3 and in the Main Study, Chapter 4

2.4 - DNA Extraction from Paraffin Embedded Tissue

Qiagen DNeasy kits (cat no 69506) were used for all DNA extractions using the following protocol.

1. 2x 15μm sections were cut from paraffin blocks from each case (for case selection see Chapter 5) and placed in a 1.5ml eppendorf tube. A clean microtome blade was used for each sample to avoid risk of contamination of tissue.

2. 2ml of xylene was added and the tube was allowed to stand for 5 minutes. Samples were then centrifuged in a microcentrifuge at 20,000 x g for 5 minutes.
3. Supernatant was then removed and 1.2ml of 100% ethanol was added. Samples were then centrifuged at 13000 rpm for 5 minutes. Supernatant was removed and this step was then repeated twice.
4. Tubes were incubated at 37°C for 15 minutes with the lid open to evaporate remaining ethanol.
5. Tissue was then re-suspended in 180µl of buffer ATL and then 20µl of proteinase K. Overnight incubation was undertaken at 56°C.
6. Samples were vortexed briefly, then 200µl of buffer AL was added, and vortexed again before 200µl of 99% ethanol was added and mixed by vortexing.
7. The mixture was pipetted into a DNeasy Mini spin column and the centrifuged at 6000 x g for 1 min. The flow-through was discarded.
8. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µl Buffer AW1 was added. It was centrifuged for 1 min at 6000 x g and the flow-through discarded.
9. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µl Buffer AW2 added. It was then centrifuged for 3 min at 20,000 x g to dry the DNeasy membrane. The flow-through was discarded.
10. The DNeasy Mini spin column was placed in a clean eppendorf tube,200 µl Buffer AE was pipetted directly onto the DNeasy membrane. It

was ncubated at room temperature for 1 min, and then centrifuged for 1 min at 6000 x g to elute.

2.5 - APOE Genotyping

The polymerase chain reaction (PCR) reaction was carried out in a total volume of 50µl of mixture. The final concentrations of the PCR reaction reagents are given in table 2.5.1.

Table 2.5.1 – Reagents for APOE PCR

Reagents	**Final Concentration**
10 x PCR Buffer minus Mg (Invitrogen)	1x
$MgCl_2$ 50mM (Invitrogen)	1.8 mM
dNTP 10mM (Invitrogen)	0.2 mM
Betaine 2.6M (sigma)	1.3 M
Primers (each)	0.1 mM
Taq DNA polymerase	1.25 units
DNA	1-5µg
H_20 to a volume of 50µl	

PCR primer sequences:

5' - ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC A – 3'

5' - TCC AAG GAG CTG CAG GCG GCG CA – 3'

The PCR reaction was carried out under the following conditions.The PCR mixture was initially denatured at 95°C for 3mins, followed by 40 cycles running at 94°C for 1min, 60°C for 1min, 72°C for 2min. A final extension was undertaken at 72°C for 8 minutes.

15µl of PCR product were then removed and run on a 3% agarose gel at 120v for 1 hour to visualise the 220bp APOE gene. The remainder of the PCR product was subjected to restriction enzyme digest using Hha1 (New England Biolabs). The digest was carried out at 37°C for 2 hours. Digested products were then run on a 4% metaphor agarose gel at 120v for 1.5 hours.

Results of DNA extraction and separation of APOE alleles is shown in Chapter 5

2.6 - Statistical Analyses

Statistical analyses were performed using the statistical software Minitab and the individual tests used are indicated in following Chapters – the Initial Study, the Main Study and the APOE study. The advice of a statistician was sought and followed on the methods used.

2.7 - Ethical Approval

All cases used in this study were anonymised. Ethical permission was sought and obtained for this study **(LREC/2003/6/6).**

Chapter 3: Initial Study

3.1 – Introduction

The focus of this initial study is the attempted quantitation of cell populations in the in the white matter of HIV negative children and their responses in HIV infection.

Normal White Matter- Anatomy and Physiology

In Chapter 1 the normal brain components were described, including the structure and function of the axon and the oligodendrocyte. Here an account of the white matter with particular reference to its organisation and the biology of myelin is presented.

In the adult human brain the white matter contributes just under 50% of the volume of the cerebral hemispheres (Miller et al. 1980). Although the demarcation between grey and white matter is largely distinct there are loci where this is blurred, as in the basal ganglia. The arrangement of the fibre tracts in the hemispheres is best displayed by blunt dissection of the formalin fixed brain. The fibres of the white matter may be considered in three main systems according to their connections and directions

1. The commissural fibres connect corresponding areas of the two hemispheres.
2. The long and short arcuate fibres connect different cortical areas within the same hemisphere.

3. The projection fibres connect the cerebral cortex with the grey matter of the basal ganglia, brain stem and spinal cord.

Cellular Components of White Matter

The linear arrays of **oligodendrocyte** nuclei are a familiar sight in routinely stained sections of central white matter tracts such as the corpus callosum. The oligodendrocyte population in normal white matter is heterogenous with three main classes described according to nuclear size and tinctorial properties – dark nuclei with a nuclear size of 3.5-5μm, nuclei of medium shade with a nuclear size of 4-7μm and light nuclei of nuclear size 6-8.5 μm(Mori and Leblond 1970). This study was based on examination of the corpus callosum of the rat. Mitotic activity could be detected in all except in dark nuclear group. Other studies have confirmed the mitotic potential of oligodendrocytes (Suzuki and Raisman 1992) and especially the capacity of fully differentiated, myelin bearing oligodendrocytes to proliferate.

The spatial correlates of oligodendrocytes were the subject of a study of the adult rat fimbria, in 1992 (Suzuki and Raisman 1992) (the fimbria is the efferent pathway of the hippocampus and contains both projection and commissural fibres). The tract was studied in both longitudinal and transverse planes and showed arrays of oligodendrocyte nuclei were more or less continuous in the long axis but interposed with astrocytes, so that a run of 8 or so oligodendrocytes would be followed by a single astrocyte. In the transverse plane these oligodendrocytes "cores" were separated from each other in a regular way and at a mean distance of 15.7μm. Each core of oligodendrocytes

was associated with a surrounding shell of fibres, estimated to number some 1,200, of which approximately 800 were myelinated. Previous studies have suggested that a single oligodendrocyte might contribute segments of myelin on up to 50 separate, but neighbouring, axons (Peters 1964) (Butt and Ransom 1989) and that most of these were at some distance, up to 40μm in some cases, from the oligodendrocyte cell body. The mean inter-node length, that is the length of each myelin segment, was estimated to be about 200μm and the width of the node (node of Ranvier – see below in section on myelin) 0.8 – 1.1μm (Hildebrand et al. 1993). Apart from the linear arrays of oligodendrocytes, these cells were also seen to run in a different direction as they accompanied capillaries in their courses through the white matter. Oligodendrocytes maintain contact through tight junctions (specialised parts of the plasma membranes of adjacent cells that are in such close contact that the extracellular space is excluded). The chief function of oligodendrocytes is to ensheath axons. For larger axons this is by means of a myelin sheath. The derivation of myelin from oligodendrocytes is covered in chapter 1. In early studies, myelin was considered to be a relatively simple and static substance composed largely of proteins and lipids. Advances in molecular biology have shown it to be more complex and surprisingly dynamic in its biology, with a relatively high metabolic rate (Persson et al. 1992).

Some 70-80% of myelin is lipid in nature and the principal lipids, which make up some 65% of the total (Norton 1984) are galactolipids, including cerebrosides and sulphatide, cholesterol and ethanolamine phosphoglyceride. Cerebroside is used as a marker probe for myelin and oligodendrocytes. The

principal proteins of myelin are myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG) and 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNP). MBP has a molecular weight (mw) of 20 kDa and is mainly associated with the intra-cytoplasmic membrane region of myelin, which corresponds to the major dense line seen in electron-micrographs. It is considered to be a major determinant of the compaction of mature myelin and mice lacking the MBP gene (mouse mutant shiverer) show defective lamellar structure of myelin with frequent splitting at the major dense line (Benjamins et al. 1984). A sequence in its molecule renders it encephalitogenic and this forms the basis of the model of experimental allergic encephalitis (EAE)(Waksman and Adams 1962). Antibodies to MPP are used as myelin markers in studies of myelin development and integrity. PLP has a molecular weight of 25 kDa, similar to MBP, and is thought to participate in securing the structure of myelin (Moscarello et al. 1973)The mutant Jimpy mouse that has a genetic defect in the gene coding for PLP manifests a severe myelin abnormality (Sprinkle 1989) PLP shares the antigenic properties of MBP (Brostoff and White 1986). MAG has a molecular weight of 100-110 kDa and is involved in cellular interactions such as adhesion between neurons and oligodendrocytes (Kucherer et al. 1987). CNP, which constitutes 2% of all myelin proteins, is thought to be involved in myelinogenesis and myelin maintenance(Braun et al. 1990). Another myelin protein, myelin-oligodendrocyte-associated glycoprotein (MOG), of molecular weight 51-54 kDa, is of interest because experimental administration of anti-MOG serum provokes demyelination (Schluesener et al. 1987).

Generally **astrocytes** possess three kinds of processes – radial, longitudinal and vascular. The radial processes, which are thick and tapering, extend in the transverse plane, i.e. at right angles to the long axis of the tract, and for distances of up to 100μm. The longitudinal processes are thin and unbranched, up to 30μm in length and run in amongst the fascicles. The vascular processes extend for variable distances to make contact with capillaries and each astrocyte may contribute one or more end-feet to a capillary The separation of astrocytes in the cores varies from 35 to 70μm. The spatial arrangement and degree of separation of astrocytes has been investigated and shown to be non-random (Distler et al. 1991), and to be determined by astrocytes alone, without reference to surrounding structures or cells (Tout et al. 1993).

The arrangement of the radial processes of astrocytes, is considered to provide a significant scaffold for the white matter (Distler et al. 1991). Communication between neighbouring astrocytes exists via gap junctions (a specialised part of the plasma membranes of adjacent cells where the surfaces are separated by a gap of 2-4 nanometres (nm) and across which protein filaments of connexion pass, allowing communication between the cytoplasm of the cells), thereby providing a form of syncytium (Suzuki and Raisman 1992). This is in contrast to the gap junctions used by oligodendrocytes. Astrocytes are intimately involved through their processes in the nodal regions of axons, where they appear to have an active role in ionic homeostasis by means of sodium and potassium channels (Hildebrand et al. 1993).

Astrocytes also make contact with oligodendrocytes through gap junctions. In health, astrocytes are key determinants of homeostasis. Through their end feet on the microvasculature they have been shown to influence blood flow (Koehler et al. 2006) and their participation in the maintenance of the blood brain barrier (BBB) is essential to its function as gatekeeper (Abbott et al. 2006). The importance of their intimate contact with synapses and their uptake of glutamate in health precludes accumulation of this neurotransmitter, that is excitotoxic in excess (Anderson and Swanson 2000). Their close relationship with neurons underscores not only essential inter-cellular signalling but also involvement in energy transfer in circumstances where oxygen/glucose delivery is sub-optimal (Pellerin and Magistretti 2004). The natural storage of glycogen by astrocytes allows emergency supplies of this energy substitute to be used by neurons and also by axons through astrocyte end-foot contact at the node of Ranvier (Brown et al. 2002).

The interdependence of oligodendrocyte, astrocyte and axon is well demonstrated in experimental studies in which disruption of axonal integrity is followed by oligodendroyte loss and astrocyte disarray (David et al. 1984).

Microglia are distributed throughout the white matter, both within the linear arrays of oligodendrocytes and elsewhere amongst the fibres, and in a peri-vascular position, where they are distinct from perivascular cells or macrophages. In health, as the CNS arm of innate immunity, they express a low level of immune of surface molecules and, like astrocytes, have a supportive role in respect of neuron physiology through their secretion of nerve growth factor (NGF)(Streit et al. 1988). Neurons interact with microglia and

can suppress activation of these cells (Neumann et al. 1996). Microglia are considered further in chapter 4.

The proportions of glia in the mature rat have been estimated to be – oligodendrocytes 70-75%, astrocytes 17.5-20% and microglia 3.5-5% (Ling and Leblond 1973). As regards the territories of individual cells, there is considerable overlap in the fields of individual oligodendrocytes and also those of astrocytes but in the case of microglia there is seen to be a strict demarcation of territory and this is supported by very recent work using micro-imaging techniques in vivo (Jinno et al. 2007).

Nervous tissue is highly dependent on a continuing supply of oxygen and glucose and the demands of white matter are scarcely less than those of grey matter. The resting cardiac output of an average sized human adult at rest is 5 litres a minute, of which the brain receives 800 mls/minute, or about a seventh. Total oxygen consumption of the brain is approximately 3.5mls per 100 gm. of brain tissue per minute. The capillary bed of the brain is not fed by end-arteries as they are in other tissues, and the brain capillaries form frequent anastomoses before uniting to form venules (Peters 1961). In this way nervous tissue is protected as far as possible from fluctuations in blood flow. However, the point is made that the benefits that the myelin sheath confers in terms of energy efficiency in the axon comes at a price, in respect of oligodendrocyte vulnerability (Hildebrand et al. 1993).

The Pathology and Pathophysiology of White Matter

Some of the reactive responses of glial cells were considered in Chapter 1 but are considered in more detail here.

Astrocytes respond to local tissue damage in a variety of ways. They may enlarge with increase in the number and length of their fibres (Norenberg 1994). This swelling occurs as an early response to traumatic injury, ischaemia, status epilepticus and hypoglycaemia among other insults. The mechanism of this swelling is complex, involving glutamate and an influx of sodium ions. Astrocyte swelling may be a stimulus for proliferation (Reichenbach 1989). which may be part of the later response of astrocytes to tissue injury. Early swelling is followed by more distinctive changes including cytoplasmic hypertrophy and nuclear enlargement with increased chromatin and more prominent or numerous nucleoli. Glial fibrillary acidic protein (GFAP) becomes a much more prominent constituent in astrocyte cytoplasm (Eng et al. 2000). Eccentric placement of the nucleus and increased expression of GFAP characterises the swollen reactive astrocytes known as gemistocytes. Occasional multinucleate forms are observed. The striking increase of long, thick cytoplasmic processes is termed gliosis. In experimental animals, gliosis follows the application of a minor stimulus within 24 hours, reaches a maximum in 3-4 days and then gradually subsides over the next 2-3 weeks (Amaducci et al. 1981). In humans the process is more protracted (Osterberg and Wattenberg 1963). The apparent increase in astrocyte numbers in gliosis may be more a factor of their size and increased expression of GFAP than of actual proliferation (Eddleston and Mucke 1993).

Reactive swelling may play a part in cerebral oedema. Astrogliosis, if extensive, can interfere with remyelination and the regeneration of damaged axons, as in multiple sclerosis. The appearance of some areas of tissue damage might suggest that astrocytes migrate into such loci but there is no good evidence that this can occur (Hatton et al. 1993).

Two particular features of astrocytic activation raised in the review by (Norenberg 1994) may be relevant to this initial study. One is the finding that diffuse reactive astrogliosis can occur in response to a focal injury (Eng and Ghirnikar 1994), possibly due to intercellular gap junction signalling. The other is that astrocytic responses may be out of all proportion to the extent of local tissue disturbance, resurrecting an idea first raised by Charcôt (Charcôt 1868) namely that astrocytic hypertrophy may be primary rather than secondary.

Oligodendrocytes have a rather restricted repertoire of reactions when compared with astrocytes. Minor ischaemic and metabolic insults are accompanied by cytoplasmic swelling but more serious disturbances are followed by cell death, either by necrosis or apoptosis. Oligodendrocytes, like neurons, and unlike astrocytes, do not contain glycogen and are especially vulnerable to injury by hypoglycaemia. Astrocytes are able to convert glycogen to lactate in this situation and thereby protect themselves and, to some extent, the axons with which they are associated (Wender et al. 2000). Oligodendrocytes are also highly susceptible to oxidative stress. They express functional glutamate receptors (Gallo and Ghiani 2000) and death occurs readily if these receptors are over-activated (McDonald et al. 1998). Cytokines,

such tumour necrosis factor alpha (TNF-α), have been shown to mediate oligodendrocyte death by apoptosis in mice (Akassoglou et al. 1998) but the same molecule promotes proliferation of oligodendrocyte precursors (Arnett et al. 2001).

Nutritional disorders affecting myelin, including excess food intake (overnutrition) and under-nutrition, can impact on CNS structure and functioning. High intake of animal fat, obesity and lack of exercise encourage the development of atherosclerosis and its attendant problems of stroke and hypoperfusion injury. Malnutrition, prevalent in the developing world, can affect central nervous system (CNS) development in the foetus of a malnourished woman, or myelination may be delayed in early childhood (Martinez 1982) Specific deficiencies, such as thiamine deficiency, can lead to microhaemorrhages in the mamillary bodies, astrogliosis and demyelination. Vitamin B_{12} deficiency, occurring in the course of malabsorption or pernicious anaemia, can result in subacute combined degeneration of the cord, in which the posterior columns and corticospinal tracts show vacuolation of myelin and demyelination.

Cerebral oedema is an important reaction occurring in response to many of the factors mentioned above. A significant degree of oedema was noted in 45 of the 156 cases (29%) of the African paediatric cohort from which the current study derives (Bell et al. 1997).

There are a number of classifications of cerebral oedema and a simple scheme is as follows:

- **Vasogenic** - in which the BBB is defective and extracellular accumulation of water, sodium and protein occurs, especially in the white matter. There is pallor of myelin with vacuolation and reactive astrocytes, including gemistocytes, are seen. If oedema persists for weeks, loss of myelin may occur especially in the periventricular white matter.
- **Cytotoxic** – the commonest cause of this type is ischaemia and the changes are mostly intracellular as a result of energy failure in neurons. Other causes of this cytotoxic oedema are toxins such as triethyl tin and hexachlorophane.
- **Hydrostatic** - in this type there is passage of protein poor fluid into the extracellular space leading an increase in intravascular pressure and dilating the capillary bed e.g. hypertensive encephalopathy.
- **Interstitial oedema** - as in obstructive hydrocephalus where fluid is forced into the periventricular white matter.
- **Hyperosmotic oedema** – in which the swelling is due to a large reduction in serum osmolality.

  (Esiri 1996)

Some dietary items in susceptible individuals may cause disease of the CNS or of the peripheral nervous system (PNS). Coeliac disease, in which there is intolerance of the protein gluten found in wheat, is occasionally accompanied by disorders of the CNS and PNS (Cooke and Smith 1966) (Wills 2000) Examination of the brain in such cases may show a variety of histological abnormalities, including cerebellar Purkinje cell loss and spongiform

demyelination in the posterior and lateral columns of the spinal cord, with lymphocytic infiltration in the hypothalamus, brain stem, cerebellum and spinal cord in some cases (Cooke and Smith 1966).

Cell Growth and Proliferation

These activities are considered in some detail to provide a background for the intriguing phenomenon of non-neoplastic cellular proliferation in tissues and organs.

The natural processes of cellular growth, proliferation, migration and differentiation, which characterise early embryonic life, soon become stable as development proceed. Cells such as neurons are considered to be terminally differentiated, unable to divide and not replaceable in the event of their death by natural ageing, trauma or disease. In the last 25 years or so there has been progressive modification of the concept of cellular differentiation because of the advances in the fields of molecular biology and genetics and, in particular, in stem cell research (Rosenthal 2003).

The dynamics of cell growth and proliferation have always been a subject of scientific interest because of their relevance in cancer research. In addition, proliferation of immune cells, and of the parenchymal cells of the target tissue(s), is often an integral part of disease processes, especially in inflammatory disorders such as rheumatoid arthritis. Cell loss by necrosis or apoptosis is no less interesting but is considered outwith the scope of this study.

The cell cycle, which provides the basis for the understanding of cell division, is now seen as a very complex process (Kumar 2004) and subject to numerous stimulatory and inhibitory influences. Although cellular growth and proliferation are sometimes discussed as though they were the same process, they are separate events: the former signifying an increase in cell size and the latter, an increase in cell numbers. The corresponding terms used in pathology are hypertrophy and hyperplasia. Though they are intimately related and growth usually precedes proliferation or mitosis, they can be independently controlled and one process can occur without the other (Su and O'Farrell 1998).

The influences which have a positive or stimulatory effect on the cell cycle are known as **growth factors** and a large number are known to be involved in normal physiology and pathophysiology. Also, a large number of naturally occurring compounds, for example the glycoproteins found in many plants and known as lectins, e.g wheatgerm agglutinin, have been shown to have a potent proliferative effect on cells in tissue culture and are perhaps better referred as **mitogens.** Growth factors are generally polypeptides and often have a spectrum of activity, which, in addition to influencing proliferation, may promote cell motility and regulation of differentiation. Many are involved in repair processes following trauma or inflammation. Some of the more important growth factors are:

- **Epidermal growth factor (EGF)** – is secreted by platelets and macrophages and is mitogenic for fibroblasts and keratinocytes.

- **Insulin-like growth factor-1 (IGF-1)** – is produced by fibroblasts and macrophages and stimulates fibroblast migration and proliferation.
- **Platelet derived growth factor (PDGF)** – is produced by endothelial cells, keratinocytes, macrophages and platelets. The isoforms of this molecule have a wide variety of actions - they are potent stimulators of mitosis for endothelial cells, fibroblasts, glial cells and smooth muscle cells and have marked chemotactic properties. They are closely involved in wound healing.
- **Transforming growth factors α (TGF-α)** – is produced by endothelial cells, T lymphocytes, macrophages and platelets. These are chemotactic for a number of cells and have proliferative influence on hepatocytes and epithelial cells. In contrast TGF-β is a growth inhibitor for epithelial cells and has a potent anti-inflammatory action.
- **Vascular endothelial cell growth factor (VEGF)** – is derived from epithelial cells, smooth muscle cells and macrophages. It is important in angiogenesis and induces endothelial cell proliferation and vascular permeability.
- **Tumour necrosis factor (TNF)** – is produced by mast cells, macrophages and T lymphocytes. It has a profusion of actions including immune system activation. High circulating levels are present in HIV infection (Odeh 1990) and it is thought to play a key role in the pathogenesis of HIVE (Wilt et al. 1995) .

- **Interleukins (IL family)** – are produced by many tissues, macrophages, mast cells and lymphocytes. These cytokines have multiple functions including chemotaxis and angiogenesis.

The Cell Cycle

In health, in those tissues having a slow rate of turnover of parenchymal cells, the majority of the cells would be in a resting G0 state. Conventionally the proliferative cell cycle is divided into 4 stages – G1, S, G2 and M. G0 signifies the quiescent or pre-cycle entry stage. Cells would enter the cell cycle only if exposed to microenvironmental or systemic stimuli, many of which are now understood. In some tissues, such as the human endometrium during reproductive life, a cyclical pattern of proliferation is under hormonal control. In other tissues the dynamics of normal cell renewal are less well known but in most tissues there a regular replacement of cells that have died naturally, although the precise determinants ensuring constancy of organ or tissue mass in health are not yet defined. The experimental model of replacement of liver tissue after partial resection is often quoted (Fausto 2000) . In disease states the details of the repair process have been much studied by simple histology and are now the subject of scrutiny by molecular biological techniques. The mechanisms of escape from control by neoplastic cells are increasingly linked to cancer associated genes that now number several hundreds. The potential for cells to proliferate in the absence of external stimuli is the major hallmark of malignancy.

In normal physiology the essential steps in the process of cell proliferation are:

- One or more growth factors binds to its specific receptor(s), usually on the cell membrane. These growth factors, signalling molecules (or ligands), may have several sources as detailed below.
- Activation of the receptor, usually transient and limited, is followed by activation of several signal transducing proteins on the inner aspect of the plasma membrane.
- The signal is transmitted to the nucleus by messenger proteins.
- Induction and activation of nuclear factors occurs, leading to DNA transcription
- The cell enters the cell cycle and proceeds to division.

(Kumar 2004)

The cell cycle itself is a sequence of elaborate and tightly regulated stages each of which is subject to stimulators and inhibitors (Ekholm and Reed 2000) . The principal molecules involved belong to a family of proteins called the cyclin dependent kinases (CDKs) and these are activated by compounds synthesised de novo, known as cyclins. In the G1 phase there is a molecular on/off switch triggered by the action of a cyclin dependent kinase, CDK4, which causes phosphorylation of a molecule – retinoblastoma susceptibility protein (RB). This critical reaction is seen as overcoming the principal barrier to cell division (Sherr and McCormick 2002).

Ligands (ligand has several meanings and here signifies a molecule, or class of molecules, that has become covalently linked to another molecule to form a

conjugate) having the potential to activate growth receptors may either be growth factors, examples of which are listed above, or molecules arising in the extra-cellular matrix (ECM). Three general modes of signalling are recognised according to the source of the ligand and the location of its target receptors:

1. **Autocrine** – in this mode of signalling cells respond to molecules that they themselves secrete, a process that is referred as an autocrine loop. This type of proliferation occurs in antigen stimulated lymphocytes.
2. **Paracrine** – this mode of signalling typically occurs between adjacent, or very nearly adjacent cells, which are commonly of a different type. An example of this is in wound repair where macrophages stimulate the proliferation of fibroblasts, by means of their secretion of growth factors.
3. **Endocrine** – in this mode, long recognized, growth or proliferative effects are produced in a tissue or organ by a molecule secreted at a distance from an endocrine source – for example follicle stimulating hormone (FSH), acting on the ovarian follicle, is produced in the anterior pituitary. Endocrine growth factors are carried from the source to the target tissue in the bloodstream. Of particular relevance to the present study is the known action of cytokines produced in systemic inflammation and acting, in effect, as endocrine agents.

TNF-α was noted above as a growth factor and reference was made to the levels of this cytokine in HIV infection. A study of particular relevance was published in 1996, in which the serum levels of several cytokines were recorded in a population of HIV positive African women (Thea et al. 1996).

Elevated levels of cytokines – IL-1, IL-6 and TNF-α- were detected in asymptomatic HIV positive women. These were significantly greater than those in women with AIDS, of whom only a small percentage showed any circulating cytokines in the circulation. None of these cytokines was detected in the HIV negative controls.

These cytokines are known to enter the CNS by passage across the BBB (Banks et al. 1995) and are expressed at low levels in the normal CNS, by perivascular macrophages, microglia and some endothelial cells (Morris and Esiri 1998) The levels are considerably increased in HIV infection, both in adults (Stanley et al. 1994) and in children (Mintz et al. 1989)

Responses of Intrinsic White Matter Cells to HIV Infection of the Brain

The main neuropathology findings in HIV/AIDS were documented in Chapter 1. Here the evidence is considered for continued HIV presence in the brain during both the asymptomatic and symptomatic phases of infection and the ways in which this might impact on the cellular population of the CNS.

The presence of HIV in the brain has been difficult in those subjects dying of unrelated causes in the pre-symptomatic stage of infection (Bell 1998). However, its presence in the brain is often inferred from the finding in the cerebrospinal fluid (CSF) of HIV antigens and antibody in asymptomatic patients (Shaunak et al. 1990) (Goswami et al. 1991). Indirect evidence of a quiescent presence in the brain before the onset of AIDS was obtained from

studies of animals infected with the closely similar simian immunodeficiency virus (SIV) (Williams et al. 2001) (Sinclair et al. 1994).

In the early 1990's, two studies detected HIV-1 DNA by PCR in the brains of HIV positive asymptomatic individuals – in 1992 in one case (Sinclair et al. 1992), and in 1996 in 17 out of 36 (An et al. 1999). HIV-1 is easily detected in the brains of many AIDS patients, and in HIVE by definition. The associated neuropathological findings, including myelin pallor, astrogliosis, microgliosis microglial nodules and multinucleate giant cells, have been described in Chapter 1. Changes occurring in presymptomatic individuals include – astrogliosis, microgliosis and low grade lymphocytic infiltrates (An et al. 1996), despite the absence of productive HIV infection. The dynamics of viral/ glial interaction are of considerable interest in attempting to explain the neuropathogenesis of HIV infection where key anatomical changes such as neuronal and axonal loss occur in the late stages of infection.

Astrocytes and HIV/AIDS

In HIVE there is an extremely abnormal molecular milieu and the origins of this are likely to be present in asymptomatic pre-AIDS cases, as noted above (An et al. 1996). The pathways to astrocyte activation in asymptomatic cases are likely to be multiple and these could be either direct, in response to circulating cytokines or HIV products including gp120, alerted through their contacts with capillaries or their cell surface pattern recognition receptors (PRR) (Farina et al. 2007), or indirect via the intervention of activated or infected microglial (An et al. 1999) or neuronal signalling (Schipke and

Kettenmann 2004) As noted earlier, astrocyte activation is characterised by an increase in cell size and GFAP content (Eng and Ghirnikar 1994). At molecular level, profound changes occur. Resting astrocytes possess a host of cell surface receptors and produce a variety of cytokines and chemokines (Ridet et al. 1997). They also produce growth factors (including neurotrophic factors that are important in neuronal and oligodendrocyte survival), for example NGF (Farina et al. 2007). In activation the expression of these receptors is increased, as is the production of most growth factors and cytokines, especially TNFα (Merrill and Benveniste 1996) and IL-6 (Morganti-Kossmann et al. 1992).

Neurons and HIV/AIDS

Neurons are not known to be infected with HIV but suffer damage and loss in HIV infection of the brain. Neuronal loss is demonstrable in HIVE and its clinical correlate is HAD or lesser degrees of cognitive disorder, which can be detected by psychometric testing (Heaton et al. 1995). Although the cause of this neuronal loss is likely to be multifactorial, activated astrocytes may contribute in a number of ways. Though astrocytes are infrequently infected, such infected cells are likely to be important numerically (by virtue of the absolute size of the astrocyte population), and are a significant factor in maintaining a reservoir of virus (Brack-Werner 1999), another cause of astrocyte activation. Neuronal integrity could be prejudiced by failure of astrocytic metabolism of glutamate and its seepage from the cell to cause neuronal cytoxicity (Ye et al. 2003). Furthermore, astrocytes exposed to HIV envelope protein gp120 produce toxic nitric oxide synthase (NOS) which could

interfere with their neuronal support function (Nath 1999). TNF-α causes proliferation of astrocytes in vitro (Selmaj et al. 1990) and is associated with astrogliosis in early asymptomatic HIV infection (Gray et al. 1992) and in AIDS in cases with a history of HIV associated dementia (HAD) (Seilhean et al. 1997). Astrocyte apoptosis may occur in parallel with astrogliosis (Sabri et al. 2003).

Oligodendrocytes and HIV/AIDS

Oligodendrocytes are thought not to be infected by HIV and only a few studies have attempted to document their numbers in this condition, perhaps because there is no good immunohistochemical marker for them. Esiri, (Esiri et al. 1991) compared the changes in the oligodendrocyte populations in 22 HIV-1 infected cases with 11 uninfected controls. 14 of the 22 HIV+ve cases had AIDS or AIDS-related complex (ARC).Oligodendrocytes were detected by immunocytochemistry using antibodies to two enzymes found in oligidendrocytes – carbonic anhydrase isoenzyme II (CAII) and 2′3′ -cyclic nucleotide 3′ -phosphodiesterase (CNPase). The findings of interest were that in the normal control brains the mean oligodendrocyte density in the white matter was 667/sq.mm (in the discussion it was acknowledged that not all oligodendrocytes were labelled). Higher numbers of oligodendrocytes were found in the white matter of HIV-1 positive cases, particularly those showing only mild myelin pallor and no giant cells, suggesting that there is slight hyperplasia of oligodendrocytes in HIV-1 infection particularly before the onset of HIVE and in the absence severe myelin pallor. Conversely,

circumstantial evidence would dictate that in HIVE , where there is myelin loss, there must almost certainly be oligodendrocyte loss (Budka et al. 1987).

In respect of the response to cytokines, there is evidence from an in vitro study that TNFα is toxic for oligodendrocytes (Tyor et al. 1992) but more recently the same cytokine was found to promote proliferation of oligodendrocyte precursors (Arnett et al. 2001). IL-2 was found to cause proliferation of oligodendrocytes in glial cell cultures (Benveniste and Merrill 1986). HIV derived molecules, particularly HIV-gp120, were found to be toxic in vitro for oligodendrocytes (Bernardo et al. 1997)

Microglia and HIV/AIDS

Microglia, through their possession of CD4 and CCR5 surface receptors, are a primary target for HIV in the CNS and are the only CNS cells capable of supporting productive infection (Gartner 2000). In the late stages of infection they are the largest group of CNS infected cells (Cosenza et al. 2002). Microglial activation can be detected in the very early stages of SIV infection (Chakrabarti et al. 1991) When activated, whether by direct infection with HIV, or indirectly by mechanisms similar to those operating in the activation of astrocytes, microglia produce a wide range of cytokines and allied molecules. In particular, some of these cytokines such as interleukin-1 (IL-1), interferon (IFN)γ and TNF-α (Nath 1999) and other molecules, such as nitric oxide (NO), superoxide anions and glutamate receptor agonist, have neurotoxic potential ((Budka et al. 1987)). In addition, microglia infected by HIV produce increasing amounts of virus and viral proteins which are neurotoxic in HIV

encephalitis (HIVE) (Smith et al. 2001). Astrocytes also modulate microglial activity (Eder and Fischer 1997). Furthermore, activated T-cells promote inflammatory responses in microglia(Benveniste et al. 2004). Priming of microglia has recently been detected as a manifestation of even minor peripheral infections (Rivest 2003)

Endothelial Cells of the CNS and HIV/AIDS

Endothelial cells, which are derived from mesoderm, have a very slow turnover in health, and replacement of individual cells may not occur more often than every ten years. TNF-α was discovered to be a powerful promoter of new blood vessel formation (Leibovich et al. 1987). and in 1990 endothelial changes were noted in the blood vessels of the white matter of patients with AIDS (Smith et al. 1990). These changes included endothelial cell hypertrophy, hypercellularity and pleomorphism. This effect of TNF-α appears to be mediated at least in part through the agency of vascular endothelial growth factor (VEGF) (Ryuto et al. 1996) and IL-1 (Li et al. 1995). VEGF is the most important factor in adult angiogenesis, promoting endothelial cell proliferation in physiological and pathological settings (Ferrara et al. 2003). The modulation of this molecule by TNF-α is considered to operate in both autocrine and paracrine pathways (Yoshida et al. 1997) Another factor implicated in cerebral endothelial pathology in HIV infection is the envelope glycoprotein gp120 which, as a circulating HIV soluble product, activates endothelial cells to express cell adhesion molecules (ICAM-1 and VCAM-1)(Stins et al. 2001). As discussed in Chapter 1, the integrity of the

endothelium as the major component of the BBB plays a pivotal part in the dynamics of CNS involvement in HIV infection (Nottet 1999). (Banks et al. 2006)

Neuropathology of the Late Stage of HIV Infection

Before the introduction of HAART, some 20-30% of HIV sufferers (without opportunistic or neoplastic complications) developed HAD (McArthur, JC 1993), characterised by variable, and often profound, memory loss, motor weakness and behavioural problems. Examination of the brain in such cases showed a constellation of findings, the major features being neuronal/axonal and glial loss, extensive demyelination, inflammation with microglial and macrophage proliferation, oedema and lymphocytic infiltration and sometimes but not always, the signs of HIVE – namely the presence of HIV containing multinucleate giant cells, macrophages and microglia(Budka 1991). The problem of correlation of the clinical features with the underlying neuropathology has been the subject of extensive enquiry – neither CNS viral load nor HIVE appeared to relate directly to HAD and the best correlate for HAD appears to be the degree of microglial and macrophage activation (Glass et al. 1995).

There has been no convincing evidence for direct neuronal infection (Trillo-Pazos et al. 2003) and the neuronal damage and loss appears to be secondary, due to the influence of toxic molecules from various sources and/or disturbed signalling from normally supportive astrocytes and microglia. The toxic molecules may arise from astrocytes (Patton et al. 2000), microglia (Mattson et

al. 2005) and macrophages (Nath 1999) as described above. Viral proteins, such as gp120 and Tat, secreted by infected cells are neurotoxic (Lannuzel et al. 1995). Neuronal apoptosis has been demonstrated in HAD (Masliah et al. 1992)(a). Lesser degrees of neuronal damage, but which nevertheless contribute to the clinical burden of cognitive impairment, are dendritic pruning (Masliah et al. 1992)(b) and synaptic loss (Everall et al. 1999).

Axonal damage in the CNS of pre-AIDS and AIDS cases became readily identifiable with the application of β-amyloid precursor protein (βAPP) immunohistochemistry and a number of studies have confirmed the degree and extent of this change (An et al. 1997) (Raja et al. 1997). The principle sites of damage were found to be in the sub-cortical white matter, the basal ganglia and the brain stem (Gray 1998). The pathogenesis of axonal damage is probably multifactorial. Although axonal damage can relate to areas of myelin pallor, βAPP positivity can exist in the absence of pallor (Raja et al. 1997) . In some studies of axonal damage in HIV infection, perivascular localisation has been described (Raja et al. 1997), but this was not confirmed by other investigators in a study of pre-AIDS cases (Giometto et al. 1997)

The presence of extensive white matter damage in AIDS has been both in adults (Price et al. 1988) and in children (Sharer et al. 1986) , affecting the central white matter principally, and characterised by myelin pallor and astrocytosis. In the absence of multinucleate giant cells inflammatory changes tended to be minimal. Focal rarefaction of myelin was described and in severe cases, diffuse and focal spongy vacuolation of the myelin was present together with oedema and, uncommonly, demyelination.

The causes of these changes, like those affecting neurons, remain a subject of debate (Bell 1998) and perhaps in time new circulating molecules other than cytokines and viral products will be discovered. In the years since the introduction of highly effective anti-retroviral therapy (HAART) there has been a reduction in the incidence of HAD but conversely, the prevalence is rising because of longer survival of treated subjects. The incidence of minor cognitive defects is also rising (Bouwman et al. 1998). In terms of neuropathology in the HAART era, novel findings include an increase in inflammation and in neurodegenerative changes in the hippocampus (Anthony et al. 2005).

Background to the Present Study

Given the known effects of HIV in the nervous system of both adults and children, some of the changes in the white matter of infected individuals are entirely predictable. A preliminary simple microscopical screening of the archival haematoxylin and eosin (HE) stained sections of all cases aged two and over in the African cohort confirmed that there appeared to be differences in white matter, particularly in nuclear densities, between HIV positive and negative cases. This screening process established a rough notion of a range of "normality" in terms of nuclear numbers but the challenge was to attempt to convert these subjective impressions into more reliable data in the form of a reproducible range of total white matter cell numbers for HIV negative cases, as a basis for evaluating HIV associated pathological changes.

The **hypotheses** forming the basis of this initial study were that:

1. There would be significant differences in glial cell numbers when HIV positive and HIV negative children were compared.
2. It was likely there would be increases in cell numbers rather than decreases, affecting particularly microglia and astrocytes.

Accordingly, the **aim** of the Initial Study was to compare white matter changes in HIV positive and negative children, in particular to attempt the quantification of glial cell numbers. A small subset of the original African cohort was selected, comprising age matched HIV positive and negative children aged four years or more, in order to minimize the confounding factor of maturing myelination in younger children.

3.2 - Materials and Methods

Ten cases were selected from the original cohort of 155 cases, including four HIV positive and four negative cases of ascending age, together with an HIV negative case at the lower and upper ends of the age range (Table 3.2.1). All selected cases were aged four years or more and only 25 of the original cohort were in this age group. Of these 25, 1 was HIV-2 positive and 10 were HIV-1 positive, of which only 3 lacked significant neuropathological changes. The constraints of age and gender matching dictated that the final selection of HIV positive cases included 1 without recorded pathology, 2 with a degree of lymphocytic infiltration and 1 with HIVE. HIV negative cases were selected on

the basis of age and gender matching as well as displaying no, or minimal, CNS pathology. The causes of death found at autopsy in the selected cases are listed in Table 3.3.2

Table 3.2.1 - Initial Study Cases with age matched HIV positive and negative cases

Case No	Age-years	Sex	HIV	Brain weight-gm.	CNS Pathological findings
C85	4	F	-	1290	none on routine examination
C61	5	F	-	1285	none on routine examination
C16	5.3	F	+	1305	none on routine examination
C48	5.8	M	-	1480	none on routine examination
C31	5.5	M	+	1180	HIV Encephalitis
C79	7	M	-	1455	microhaemorrhages
C80	7	M	+	1555	lymphocytic infiltrate only
C40	8	M	-	1310	microhaemorrhages
C146	8	M	+	1375	lymphocytic infiltrate only
C84	9	M	-	1670	microhaemorrhages

Key to Table 3.2.1: Where the age is not a whole number, the number after the decimal point signifies an approximate fraction of a year as determined in the original study, based on somatic length measurement (Lucas et al. 1996). This derivation is acknowledged to be a rather imprecise estimate of age

because of the possible influences of malnutrition and disease on growth, both in fetal and post-natal growth.

Table 3.2.2 – Causes of death, based on autopsy findings, in the Initial Study cases

Case	Cause of death	Associated conditions
C85	enteritis	past malaria
C61	enteritis	
C16	interstitial pneumonia	CMV enteritis
C48	anaemia, profound	haemolytic uraemic syndrome
C31	pneumonia, bilateral	
C79	leukaemia	pancreatitis
C80	sickle cell anaemia	colitis
C40	TB - generalised	
C146	pneumonia measles	enteritis
C84	lymphoma B-cell	

Four different brain areas were chosen in the selected cases, including the cerebral convexity, the hippocampus, the basal ganglia and the cerebellum. Sections were cut from the appropriate blocks and stained with haematoxylin and eosin (HE), Luxol fast blue (LFB) and immunostained for glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), Beta amyloid precursor protein (βAPP) and von Willebrand factor. Stained sections were used to assess the presence of white matter changes in myelin, axons and vascular

endothelium. Sections stained with HE and immunostained for GFAP were used in the counting process and the results recorded using a manual tagging system. A systematic, i.e. non random, approach was adopted with respect to the areas of white matter sampled, as it is known that different white matter tracts differ in their numbers of associated oligodendroglial cells (Suzuki et al. 1988). Sections were examined blinded to age, sex and HIV status.

The plan was to sample white matter within the chosen section where the preferred orientation of fibres was in longitudinal, i.e. linear or curvilinear, array. However despite careful examination of previously stained sections from the original study, and the blocks themselves, it was apparent that this was not possible for all cases because of the variation of original sampling at post-mortem or that the requisite blocks were not available. Within the internal capsule the oblique cut of fibres dictated a departure from this plan but contrary to expectations satisfactory results were obtained. In the case of the cortical sections, because of varying availability, fronta l or parietal cortex was used.

In the cortical sections, areas of white matter were selected in the short arcuate fibres (CNS region 1), in the core of one or more gyri (CNS region 1a), and in the core of the parahippocampal gyrus (CNS region 2), as well as in the fasciculi of the internal capsule (CNS region 3) and at the bases of the folia in the cerebellum (CNS region 4). The cells of the white matter were provisionally identified by nuclear morphology in the case of oligodendrocytes, microglia and endothelial cells and GFAP staining for astrocytes. Total nuclear numbers, and subsequently the subsets of glial cells

according to their GFAP positivity or nuclear morphology, were counted. Early attempts to also count inflammatory cells, particularly lymphocytes, were abandoned because of variability in the quality of immunostaining for lymphoid and macrophage markers. The reasons for this failure were at first obscure despite attempts to optimize methods. However in the Main Study, success was achieved using new antibodies and new coverplates for each new staining run. Hence the initial failure was attributed to less than optimal reagents.

Using sections stained with HE and for GFAP, two separate and complete cell counts were made. The first count, an exploratory exercise, was based on a total field sampling area for each section of 1 sq. mm, accomplished by taking 12.33 fields, amounting to a total area of 1 sq.mm. This proved time-consuming and inefficient and, after an informal discussion with a stereologist, a further count was made, based on a smaller sample of fewer fields, total area of 20243.8. sq.μm. When multiplied by 4.94, the second set of totals achieved total areas of 1 sq.mm, matching the first set of data. These two counting assessments are set out in **Appendix 7**. The means of the two counts were determined and the values so obtained were the ones used in subsequent calculations.

Sampling was generally spaced as widely within each zone as possible. In the case of the cerebellum, for example, fields were sampled from the bases of separate folia, and in the internal capsule, one from each fascicule, if there were sufficient. The cell counting rules were fairly simple – all nuclei in focus, other than those having an overtly neuronal morphology, were counted in

sections stained with glial fibrillary acidic protein (GFAP) antibody and counterstained with haematoxylin.

Subsets of this total population were counted on the following basis:

1. All cells which had GFAP positive cytoplasm.
2. All cells, which by position or association, were clearly endothelial cells.
3. All cells having round or oval nuclei, irrespective of nuclear size or density.
4. All cells having elongated nuclei, equivalent to rod-like cells – where the major axis appeared twice as long or more, than the minor axis, or where the nucleus was of aberrant, for example triangular, shape.

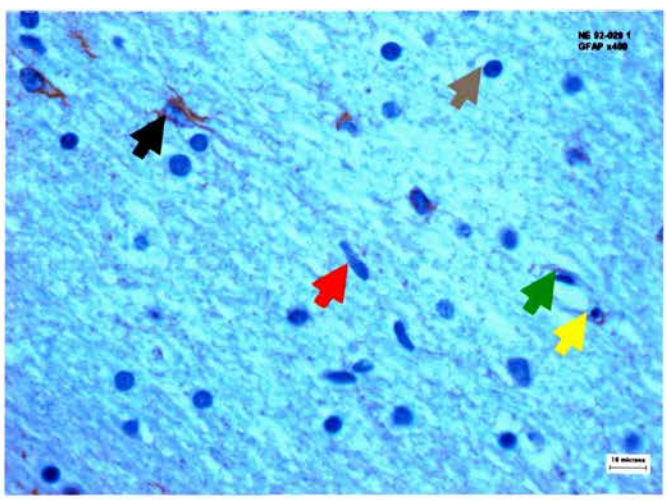
It should be added that several short trials were made to see if the counting could be semi-automated by labelling nuclei, using the soft-ware, according their area, diameter, density or degree of roundness. Though it was possible to obtain results, these almost always required adjustment by virtue of differing staining intensity between sections, inter-cell contact and out-of-focus profiles. This possible approach was therefore discarded.

The population of GFAP positive cells was considered to contain most of the fibrous astrocytes. These cells generally were characterised by having larger, lighter and polygonally shaped nuclei (see Fig. 3.2.1). The pattern of GFAP positivity varied from case to case: in some it was lightly and regularly distributed throughout the cell, and in others it appeared as a coarse particulate deposit in the processes, giving a tracer-like appearance. The presence of oedema seemed to favour this latter display.

Endothelial cells were counted if the nuclei were clearly part of a capillary, either in transverse or longitudinal section. There was little variation in their morphology, except in degree of compactness.

The population of rectangular nuclei was thought to capture a consistent proportion of the resting or ramified microglia. By adopting the practice of sampling in linear or curvilinear fibre arrays, in which the microglia lie preferentially along the axis of the fibres, their identification was more certain. Generally their nuclei were dark, but the morphology varied from rectangular to comma-like or triangular, and all these were counted together.

Fig. 3.2.1 Cerebellar White Matter stained for GFAP – subsets of nuclei for quantitation:



White matter from Case C85 (HIV negative) showing glial cells. Black arrowhead - GFAP stained astrocyte; green – endothelial cell; brown – round nucleus – possible oligodendrocyte; red – rectangular nucleus thought to be a microglial cell; and yellow – a perivascular macrophage. Original mag. x 400

Image analysis software, Image-Pro 4.5 supplied by MediaCybernetics and coupled to an Olympus BX40 microscope, was used to capture images, using the x40 objective lens. At this magnification the single frame dimensions were: width = 164.18μm, height = 123.29μm and area = 20,243.8 sq.μm. Four categories of nuclei , as shown in Figure 3.2.1, were counted using the software manual tagging "class" system. It was found easier to count starting with the most numerous nuclear populations, and the first class number assigned by the software was "0". Accordingly, in some tables there is reference to these classes and the key is:

Class 0 refers to round or oval nuclei

Class 1 refers to GFAP positive cells

Class 2 refers to rectangular nuclei

Class 3 refers to endothelial cells.

The rules were that nuclei had to be in focus, or very nearly so, to be counted. Neurons, or cells suspected of being neurons, were excluded if encountered in white matter. Nuclei in contact with the left or bottom sides of the frame were counted, those in contact with the right or top sides were not. The nuclei of cells in a perivascular location were sorted according to the above criteria and counted with the parenchymal cells.

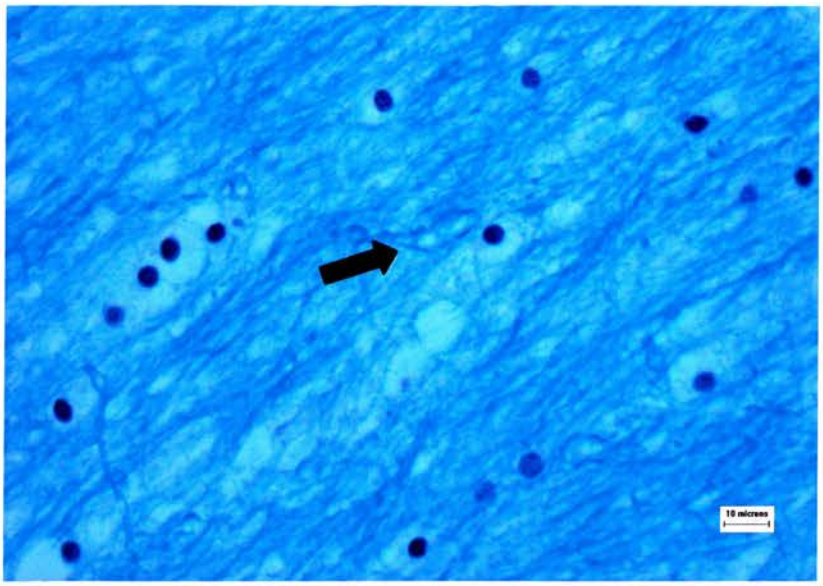
The results were tabulated in Excel for the different regions, and compared for HIV negative and positive cases using the statistical software Minitab. The data were analysed using the paired-t test (on the advice of a statistician).

3.3 - Results

Histological Assessment

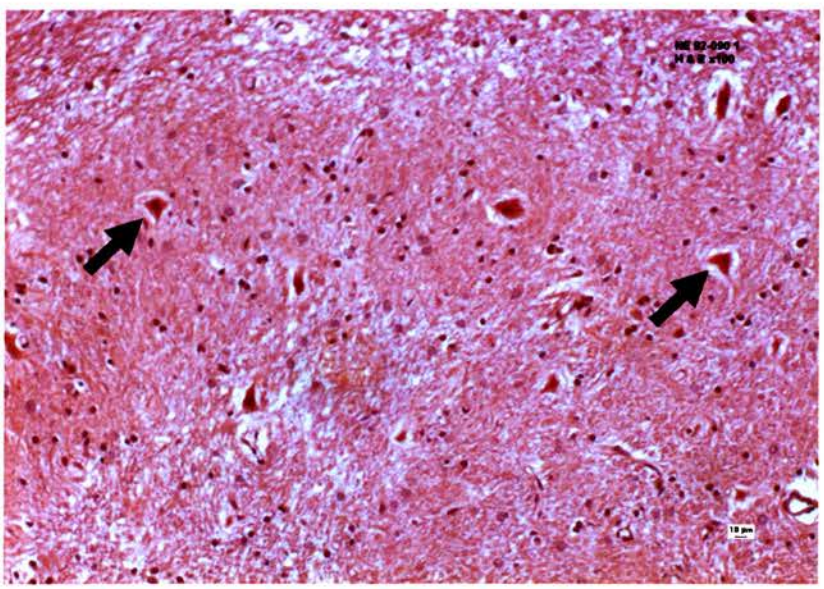
Assessment of the white matter status in routine and immunostained sections, using a light box initially, showed only one case (**c31**) was considered to show diffuse pallor in the cerebrum with respect to the others, and only one (**c84**) to show focal pallor in an area of several sq. mm. (Fig. 3.3.1). At microscopical examination, all cases were considered to show at least focal hypoxia in some if not all areas (Fig. 3.3.2). Oedema, focal or diffuse, cortical or in white matter, was a very common finding, especially in the cerebellum. In some oedematous areas pairs of astrocyte nuclei were present, suggesting a possible proliferative response (Fig. 3.3.3).

Fig. 3.3.1 Cortex Parasaggital Section



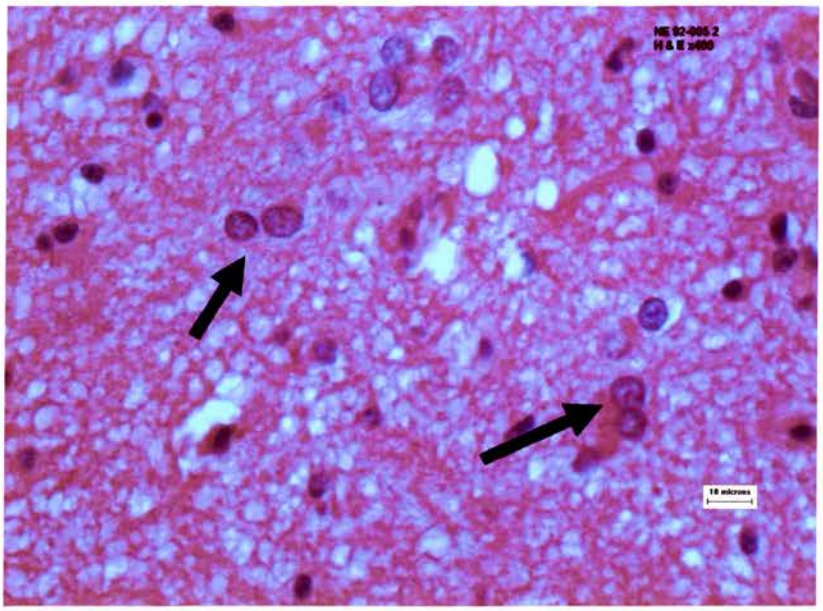
An HIV negative case (**c84**) showing focal pallor of the white matter with myelin bulb formation in the centre of the field. LFB x 40

Fig. 3.3.2 Cerebellum



An HIV negative case (**c79)** showing hypoxic neurons of the dentate nucleus. HE x100

Fig. 3.3.3 Basal Ganglia white matter



HIV negative case (**c61**) showing astrocyte nuclei in pairs in oedematous white matter. HE x 400

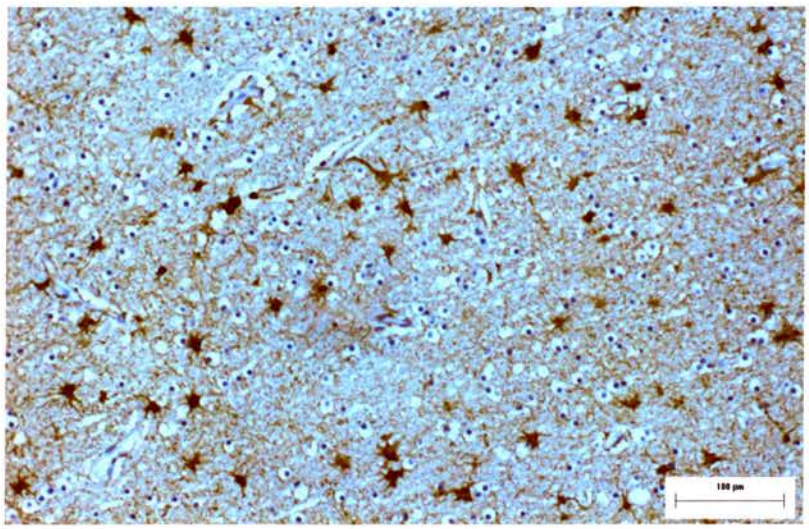
Focal abnormalities included microhaemorrhages (**c40** and **c146**) and an occasional gliotic area, for example in case **c79**. Two cases showed attenuation of the white matter in the lateral angle of the lateral ventricle (**c146** and **c84**). Perivascular macrophages and lymphocytes were more common in the HIV positive cases.

LFB stained sections were quite variable in the intensity of overall staining. When viewed on the light box there was both intra- and inter-case variation and the simple scoring system – where 1 = "normal intensity" and 2 = "slight or definite pallor" – suggested that HIV negative cases were as likely to show pallor as HIV positive cases, if not more so. The pallor noted in haematoxylin-

stained section **c31**, cortex, was confirmed in its LFB counterpart. In the same way, the pallor in the lateral angle of the ventricle in case **c146**, parasagittal section, showed staining deficiency in the LFB section.

Sections stained with GFAP showed a variety of appearances and astrogliosis was apparent in some cases, for example in the case with HIVE **(c31)** (Fig. 3.3.4).

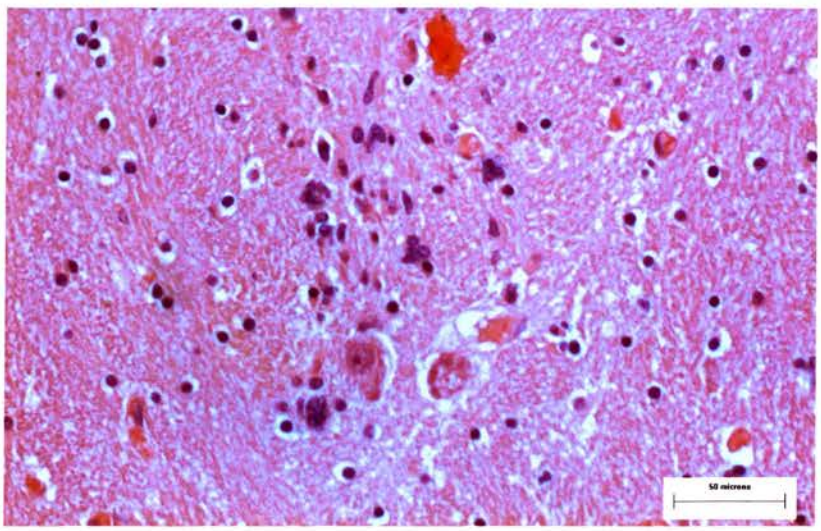
Fig. 3.3.4 Cerebral Convexity White Matter



Case of HIVE (**c31**) showing astrogliosis. GFAP x100

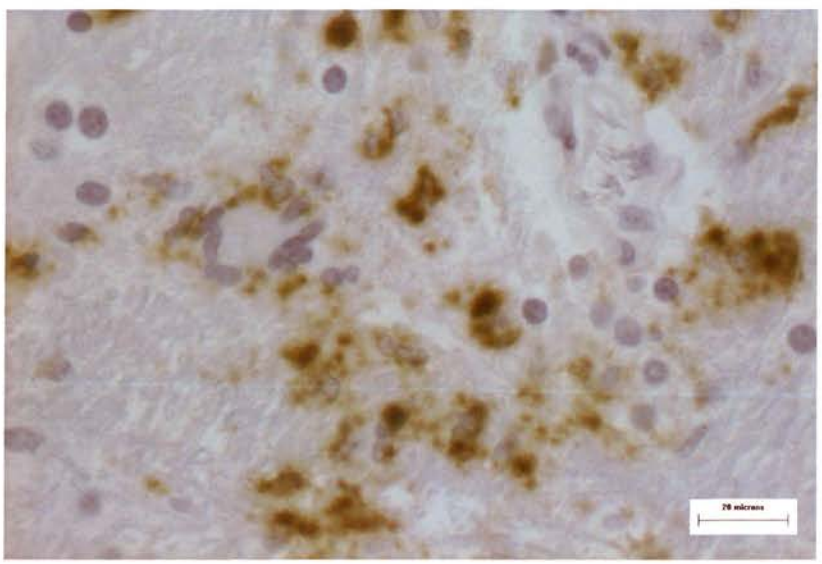
Immunostaining for HIV nucleocapsid protein p24 gave positive results for three out of the four HIV positive cases. In case **c16,** rare positive cells were seen. In the case with HIVE (**c31**), displaying giant cells (Fig 3.3.5), numerous HIV p24 positive foci were seen in the white matter (Fig 3.3.6). A single tiny focus was seen in the basal ganglia section of case **c80.**

Fig. 3.3.5 Subcortical White Matter



Case of HIVE (c31) showing a microglial nodule. HE x200

Fig. 3.3.6 Basal Ganglia



Case of HIVE (**c31**) showing a microglial nodule with a giant cell and abundant HIV p24 positivity. HIV p24 x 400

The anti-MBP stained sections were similar in display to LFB stained sections, and no additional information was obtained. Similarly no additional information was derived from the transferrin stained sections. In these sections only about one third of cells having a nuclear morphology suggestive of oligodendrocyte type were transferrin positive and, furthermore, cells with small amounts of cytoplasm and nuclei resembling those of astrocytes were also positive.

βAPP immunopositivity was infrequent and meagre. Two cases, both HIV negative (**c48** and **c79)** showed no staining at all. In most cases, affected areas were faint and only present in one or two regions. In others, restricted neuronal positivity was present, especially in the dentate neurons that had shown hypoxic change in the HE stained sections. The case (**c80**) showing most marked positivity, in all regions, was HIV positive and displayed lymphocytic infiltrates. The changes seen in the HIVE case (**c31**) were slightly less pronounced.

Immunostaining for von Willebrand factor gave generally satisfactory results. The endothelial cells varied slightly in their morphology – there was a tendency towards minor degrees of swelling in the HIV positive cases. No positive cells were seen with Ki67 immunostaining.

Cell Counting Studies

The basis for distinguishing nuclear appearances has been described. On counting the round nuclei, the presence of different nuclear sizes and densities was confirmed, as described by (Mori and Leblond 1970). They ranged from

3.5 to 8μm in diameter but no attempt was made to estimate the proportion of the different sizes.

First and second nuclear counts for each of the five white matter areas in each of the 10 cases, comprising total numbers of nuclei, nuclei of GFAP positive cells, endothelial nuclei, round and rectangular nuclei, are shown in the Table at **Appendix 8**, tabulated in Excel. Neither set of counts was uniformly greater or smaller with respect to the other. Appendix 8 also displays the means and standard deviations for the consecutive counts. In the Table, the 10 Initial Study cases are listed across the top in ascending age order, irrespective of HIV status.

The mean values of the first and second counts for each of the different brain areas, and for each of the nuclear count subsets, are set out in a two part Table (**Appendices 9a & 9b**). Appendices 9a and 9b also include an overall brain mean value, with standard deviation and paired t-test for each nuclear subset. In Appendix 9a, the results for total counts, totals minus endothelial cells and GFAP positive cells are shown. Appendix 9b shows the mean counts for endothelial cells, round nuclei and rectangular nuclei. The results shown in this two part table were used as the basis for all further analyses.

Tables 3.3.1 to 3.3.6 show summary statistics for the cell counts for cell subsets.

Table 3.3.1: Paired T-Test and CI: HIV-ve and HIV+ve mean values for total cell counts

	N	Mean	SD	SEM
HIV-ve	20	874.60	127.37	28.48
HIV+ve	20	1159.60	223.58	49.99
Difference		-285.00	202.991	45.39

P-Value = **0.000**

Table 3.3.1 shows the values for the total nuclear counts (glial plus endothelial) in all areas, for HIV positive cases and HIV negative cases separately.

Table 3.3.2: Paired T-Test and CI: HIV-ve and HIV+ve mean values for totals minus endothelial cells

	N	Mean	SD	SEM
HIV-ve	20	843.95	124.79	27.90
HIV+ve	20	1125.05	224.13	50.12
Difference		-281.10	209.111	46.759

P-Value = **0.000**

Table 3.3.2 shows the values for the total glial cell counts in all areas for HIV positive cases compared with those of HIV negative cases.

Table 3.3.3: Paired T-Test and CI: HIV-ve and HIV+VE mean values for GFAP positive cells

	N	Mean	SD	SEM
HIV-ve	20	57.60	14.7841	3.3058
HIV+VE	20	82.20	24.4295	5.4626
Difference		-24.60	24.2105	5.4136

P-Value = **0.000**

Table 3.3.3 shows the values for GFAP positive cell counts in all areas for HIV positive cases compared with those of HIV negative cases.

Table 3.3.4: Paired T-Test and CI: HIV-ve and HIV+ve mean values for endothelial cells

	N	Mean	SD	SEM
HIV-ve	20	30.5500	8.0686	1.8042
HIV+ve	20	38.1500	9.2411	2.0664
Difference		-7.6000	3.72743	1.66696

P-Value = **0.007**

Table 3.3.4 shows the values for the endothelial cell counts in all areas for HIV positive cases compared with those of HIV negative cases.

Table 3.3.5: Paired T-Test and CI: HIV-ve and HIV+ve mean values for round nuclei

	N	Mean	SD	SEM
HIV-ve	20	754.05	115.151	25.749
HIV+ve	20	971.05	200.173	44.760
Difference		-217.00	187.660	41.962

P-Value = **0.000**

Table 3.3.5 shows the values for the round nuclei cell counts in all areas for HIV positive cases compared with those of HIV negative cases.

Table 3.3.6: Paired T-Test and CI: HIV-ve and HIV+ve mean values for rectangular nuclei

	N	Mean	SD	SEM
Rect nuclei HIV-	20	33.10	19.1419	4.2802
Rect nuclei HIV+	20	71.95	36.5592	8.1749
Difference		-38.85	40.7861	9.1200

P-Value = **0.000**

Table 3.3.6 shows the values for the rectangular nuclei cell counts in all areas for HIV positive cases compared with those of HIV negative cases.

Table 3.3.7: Percentage increases in mean cell counts (total number and individual subsets) in HIV+ve compared with HIV-ve brains

For total cell counts - means of 20 values	+ 33%
For total counts minus endothelial cells - means of 20 values	+ 33%
For GFAP positive cells - means of 20 values	+ 43%
For endothelial cells - means of 20 values	+ 25%
For round nuclei - means of 20 values	+ 29%
For rectangular nuclei - means of 20 values	+ 117%

Table 3.3.7 shows the percentage increases in all cell classes in HIV positive compared with HIV negative cases.

In the next analyses, the youngest (**c85**) and oldest (**c84**)HIV negative cases were first excluded from the mean cell counts of the remaining 4 HIV negative cases and then separately compared with those means (Tables 3.3.8 to 3.3.13). In summary, in **c85** all cell types except those with round nuclei are significantly increased with respect to the corresponding HIV negative means. In contrast, when **c84** was compared with the HIV negative means in the same way no significant difference was detected for any of the cell count categories. Further detail of the means of the HIV negative cases is shown in **Appendix 10** which includes a graphical display of the degree of variation across the different brain areas for the different cell subsets (endothelial counts excluded). The percentages of each cell category are given. There seems to be reasonable accord between the populations within different brain locations, average percentages of 90%, 6%, and 4 % for the round nuclei, GFAP positive and

rectangular nuclei respectively. Although there is an impression that cell densities might be greater at convexity level than lower in the neuraxis, there is insufficient data to substantiate this possibility. An HIV negative case, **c40**, showed the least variation between cell counts in different locations.

Table 3.3.8: Paired T-Test and CI: 4 HIV-ve compared with c85 for total nuclear counts

	Mean	SD	SEM
4 HIV-ve	874.60	74.30	33.23
c85	1071.80	126.14	56.41
Difference	-197.20	151.463	67.736

P-Value = **0.044**

Table 3.3.8 shows the values for case **c85** of total cell counts in all areas compared with those of HIV negative means.

Table 3.3.9: Paired T-Test and CI: 4 HIV-ve compared with c85 for total minus endothelial cells

	Mean	SD	SEM
4 HIV-ve	844.20	74.73	33.42
c85	1033.60	134.35	60.08
Difference	-189.400	152.671	68.276

P-Value = **0.050**

Table 3.3.9 shows the values for case **c85** of total glial cell counts minus endothelial nuclei in all areas compared with those of HIV negative means.

Table 3.3.10: Paired T-Test and CI: 4 HIV-ve compared with c85 for GFAP positive cells

	Mean	SD	SEM
4 HIV-ve	57.60	11.4586	5.1245
c85	94.00	15.4434	6.9065
Difference	-36.40	7.3689	3.2955

P-Value = **0.000**

Table 3.3.10 shows the values for case **c85** of GFAP positive cell counts in all areas compared with those of HIV negative means.

Table 3.3.11: Paired T-Test and CI: 4 HIV-ve compared with c85 for endothelial cells

	Mean	SD	SEM
4 HIV-ve	30.80	3.8341	1.7146
c85	41.00	8.1548	3.6469
Difference	-10.20	5.4498	2.4372

P-Value = **0.014**

Table 3.3.11 shows the values for case **c85** of endothelial cell counts in all areas compared with those of HIV negative means.

Table 3.3.12: Paired T-Test and CI: 4 HIV+ve compared with c85 for round nuclei

	Mean	SD	SEM
4 HIV+VE	754.0	74.790	33.447
c85	851.2	149.279	66.760
Difference	-97.2	157.4554	70.4162

P-Value = 0.240

Table 3.3.12 shows the values for case **c85** of round nuclei counts in all areas compared with those of HIV negative means.

Table 3.3.13: Paired T-Test and CI: 4 HIV-ve compared with c85 for rectangular nuclei

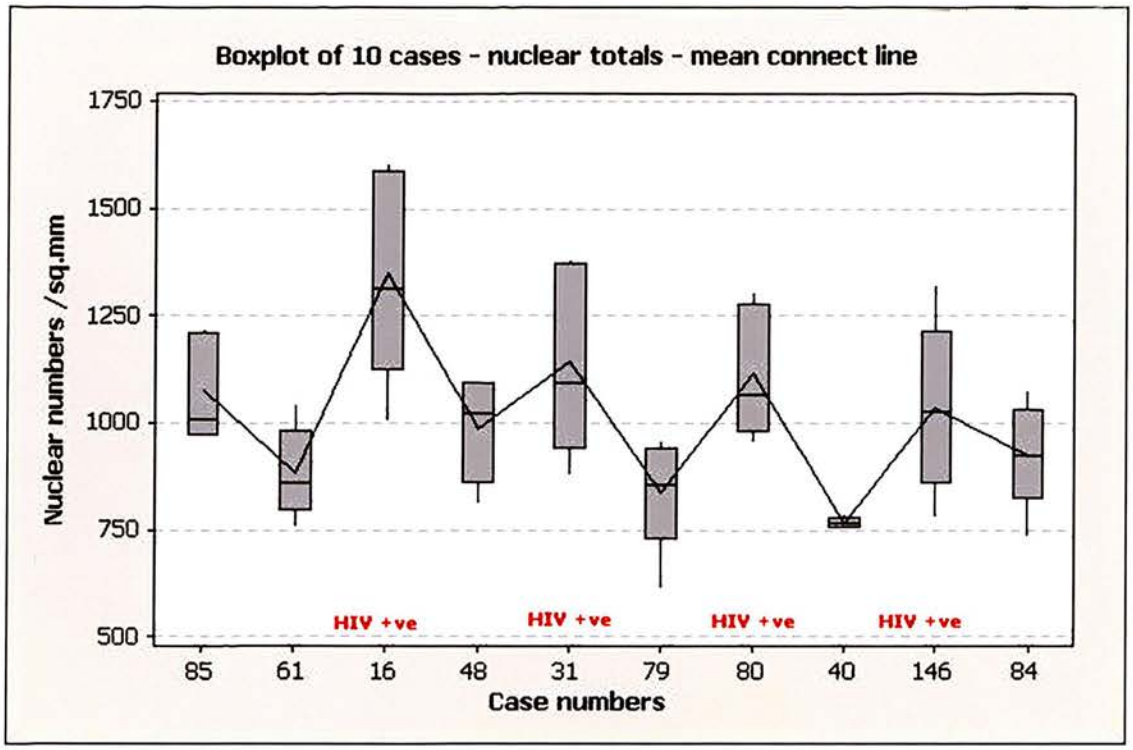
	Mean	SD	SEM
4 HIV-ve	33.20	8.5264	3.8131
c85	86.80	15.8019	7.0668
Difference	-53.60	8.1731	3.6551

P-Value **= 0.000**

Table 3.3.13 shows the values for case **c85** of rectangular nuclei counts in all areas compared with those of HIV negative means.

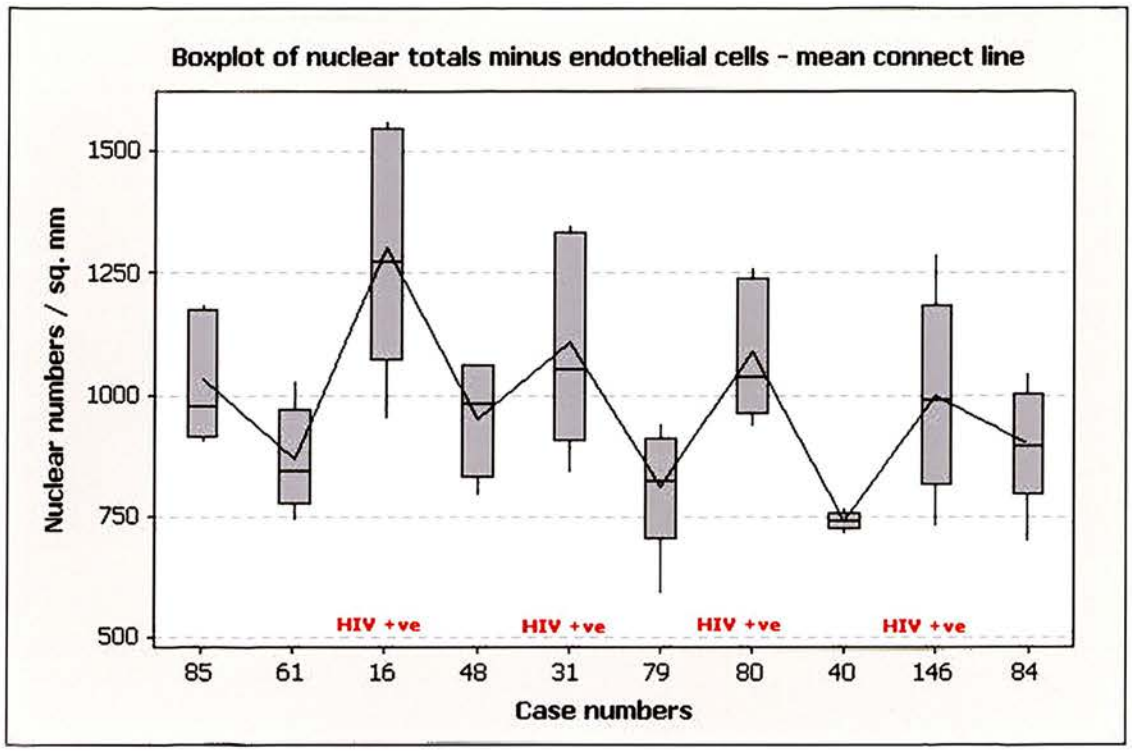
The cell counts are also illustrated graphically as boxplots which include all ten cases, including 4 age matched HIV negative and 4 positive cases, and the 2 "bookend" HIV negative cases (Figures 3.3.7 to 3.3.12). In these Figures the interquartile range boxes are arranged from left to right with case numbers corresponding to the order shown in Tables 3.2.1 and 3.2.2. The cases at either side, **c85** and **c84**, are the youngest and oldest cases, both HIV negative, and the data for these two cases are not used in the formal statistical analyses (apart from the comparison of each with the mean results of the HIV negative cases, as described in the text and, for **c85,** shown in Tables 3.3.8 to 3.3.13). The main statistical analyses are restricted to the 4 remaining HIV negative cases (**c61, c48. c79** and **c40)** and the 4 HIV positive cases (**c16, c31, c80** and **c146**). The HIV positive cases are clearly identified and a mean connect line is drawn. Each box represents five values – those of the counts in the different areas for each case.

Fig. 3.3.7



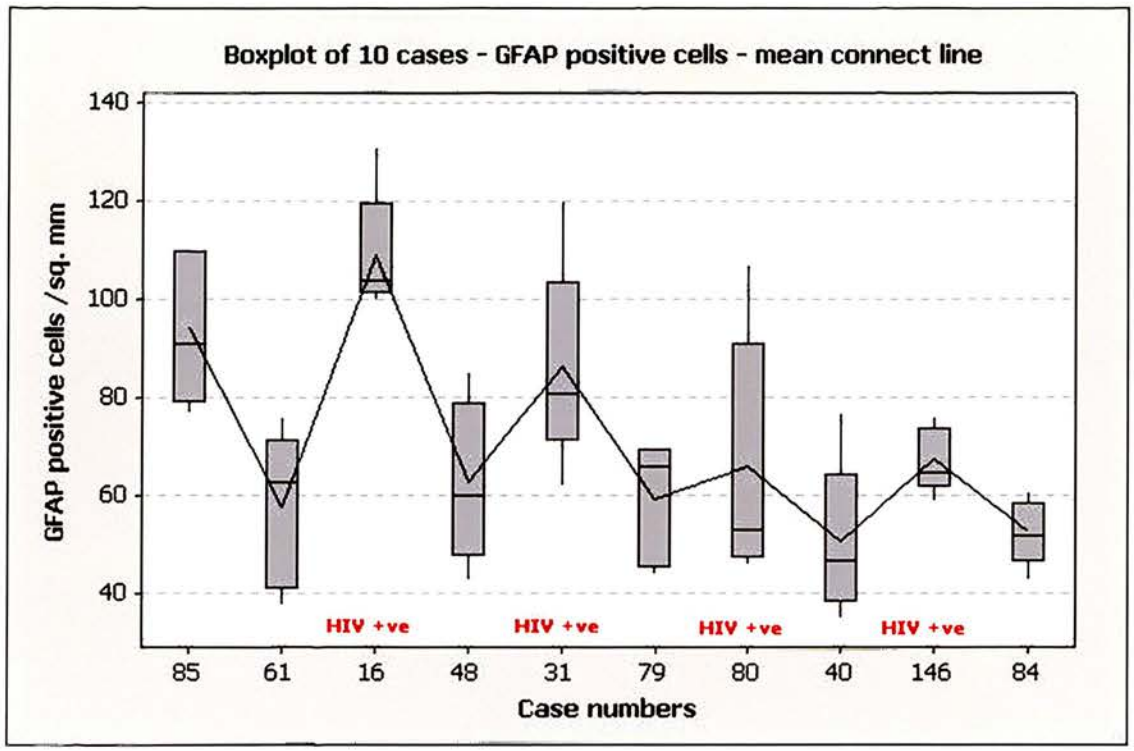
Boxplot showing nuclear totals (glial and endothelial cells) in each CNS area sampled. The HIV negative cases **c85** (age 4) and **c84** (age 9) serve to bracket the other HIV negative cases and all of the positive cases.

Fig. 3.3.8



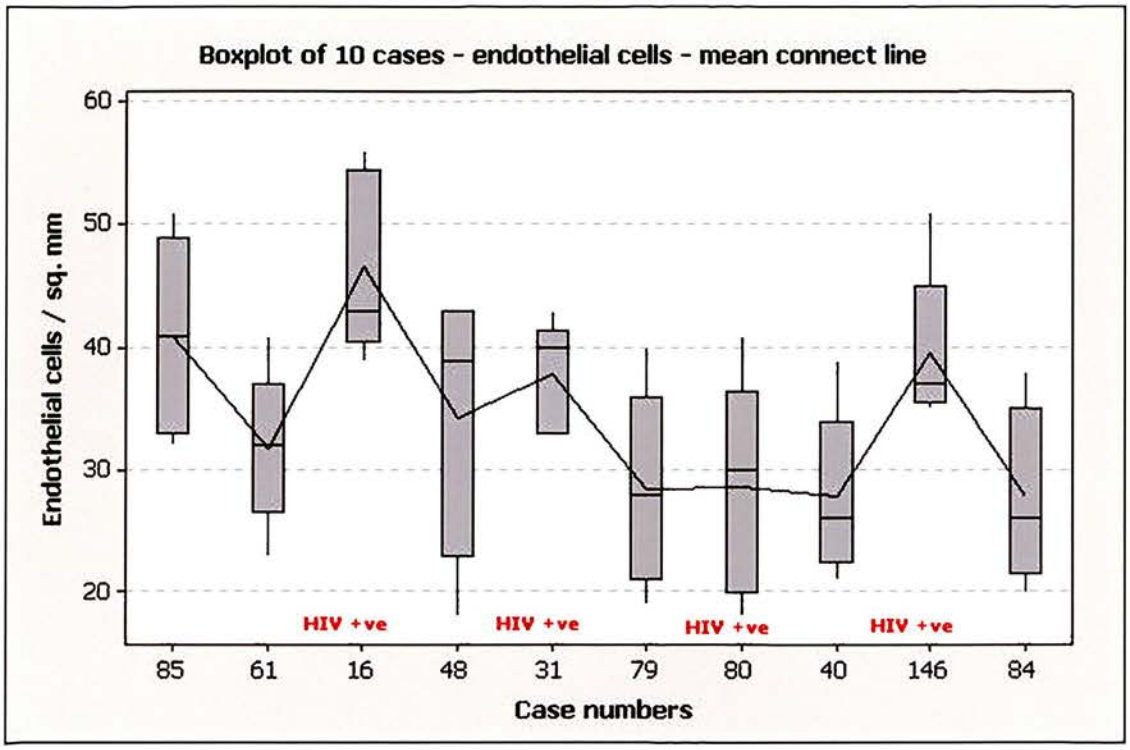
Boxplot of 10 cases – nuclear totals minus endothelial cells. These totals are for glial cell nuclei only.

Fig. 3.3.9



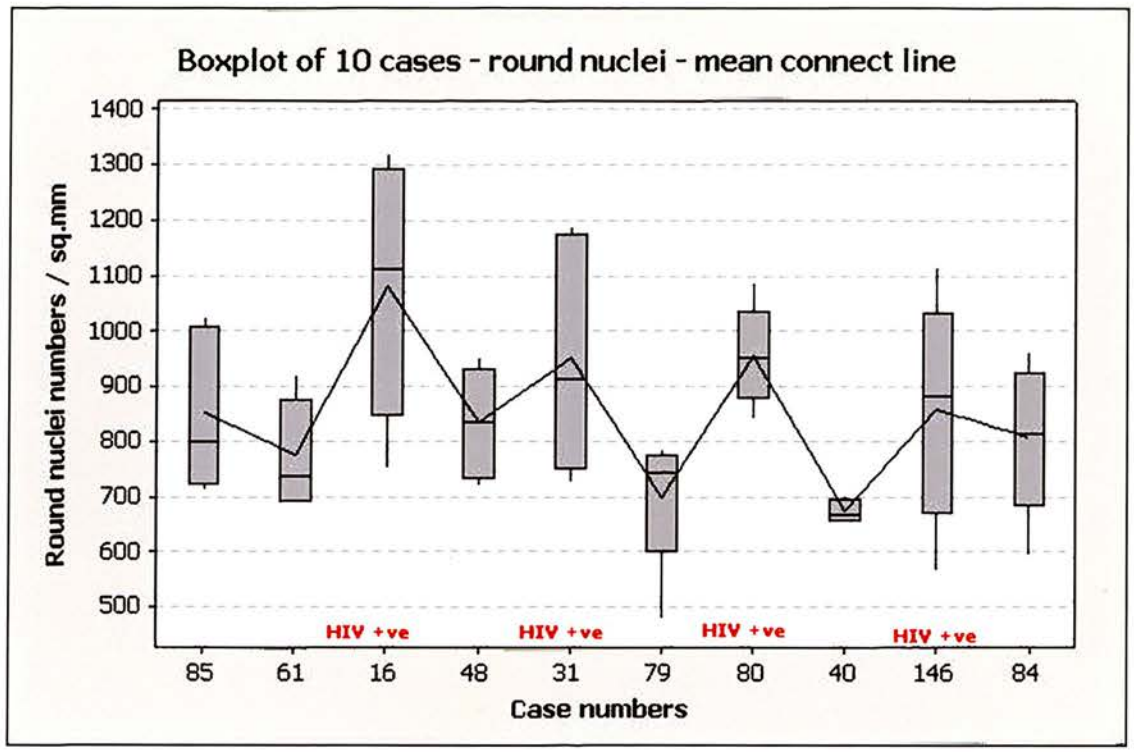
Boxplot of 10 cases – GFAP positive cells. The counts for the HIV positive cases are clearly greater than their HIV negative counterparts. HIV negative case **c85** has counts in the higher HIV positive range.

Fig. 3.3.10



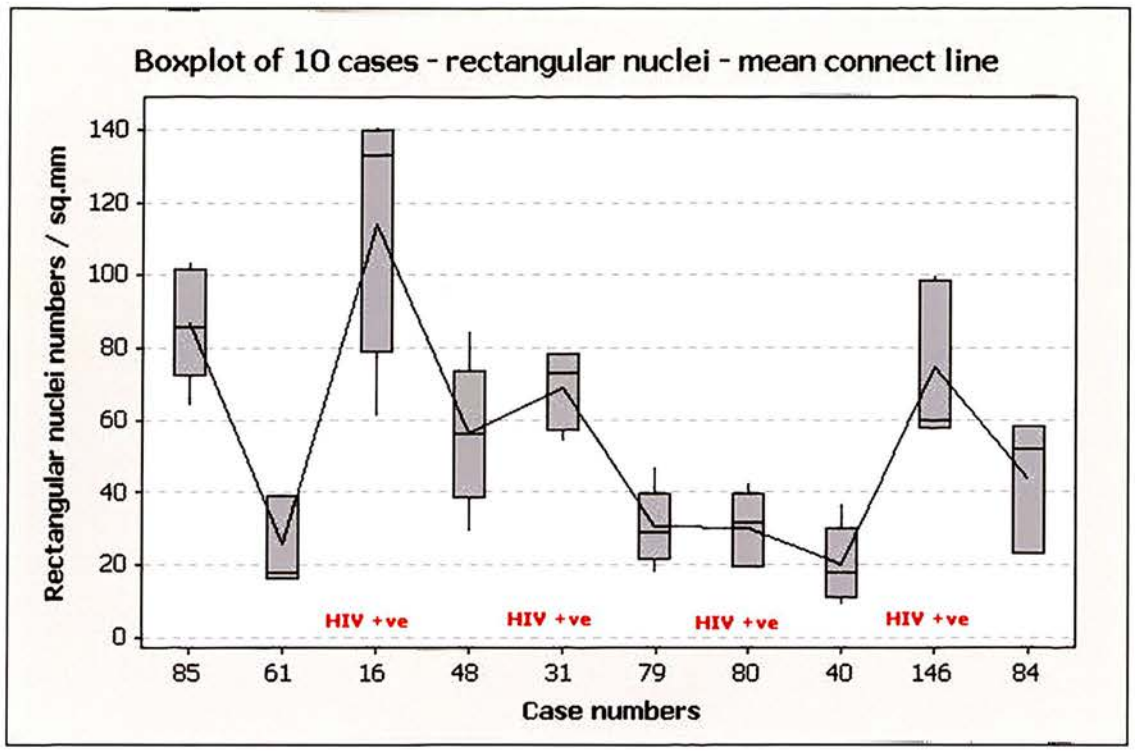
Boxplot of 10 cases – endothelial cells. The counts for HIV positive cases are generally higher than HIV negative case, **c85**, which has a higher mean and median values than the other HIV negative cases.

Fig.3.1.11



Boxplot of 10 cases – round nuclei. The counts for the HIV positive cases are higher than their HIV negative counterparts. Case **c40** shows minimal variation in count totals for the different CNS areas (mean 673 SD ±14.36)

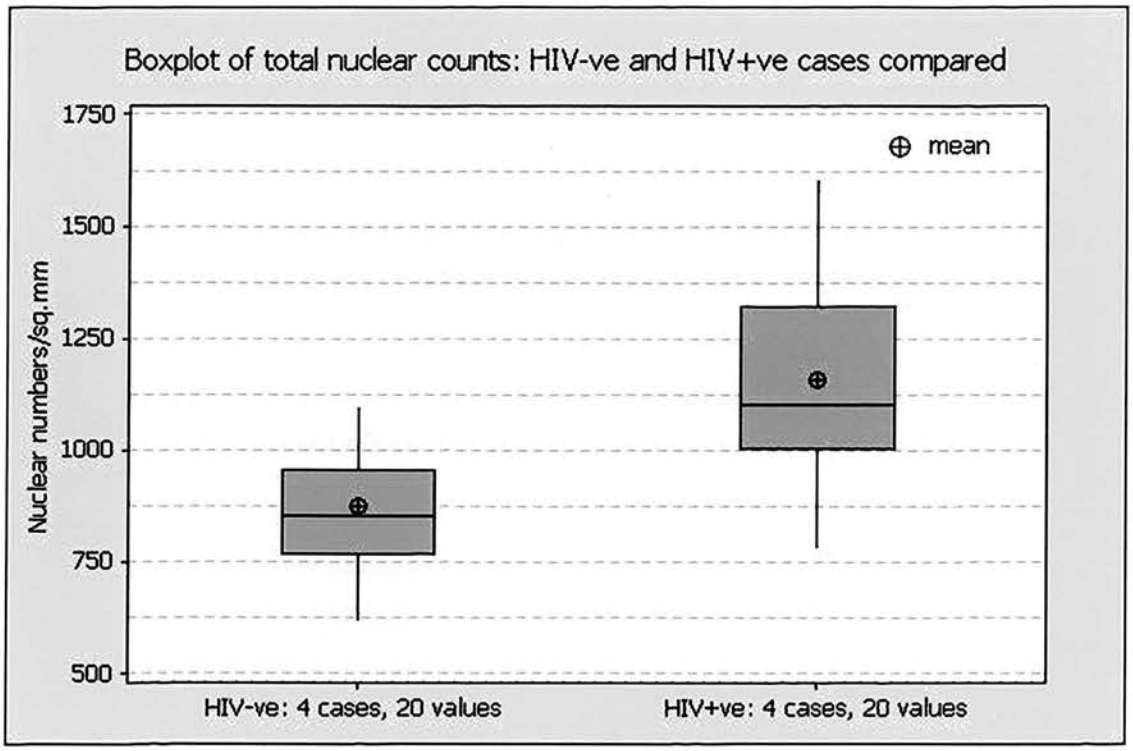
Fig. 3.3.12



Boxplot of 10 cases – rectangular nuclei. The differences between cases **c79** (HIV negative) and **c80** (HIV positive) are minimal. Case **c85** shows the second highest count overall.

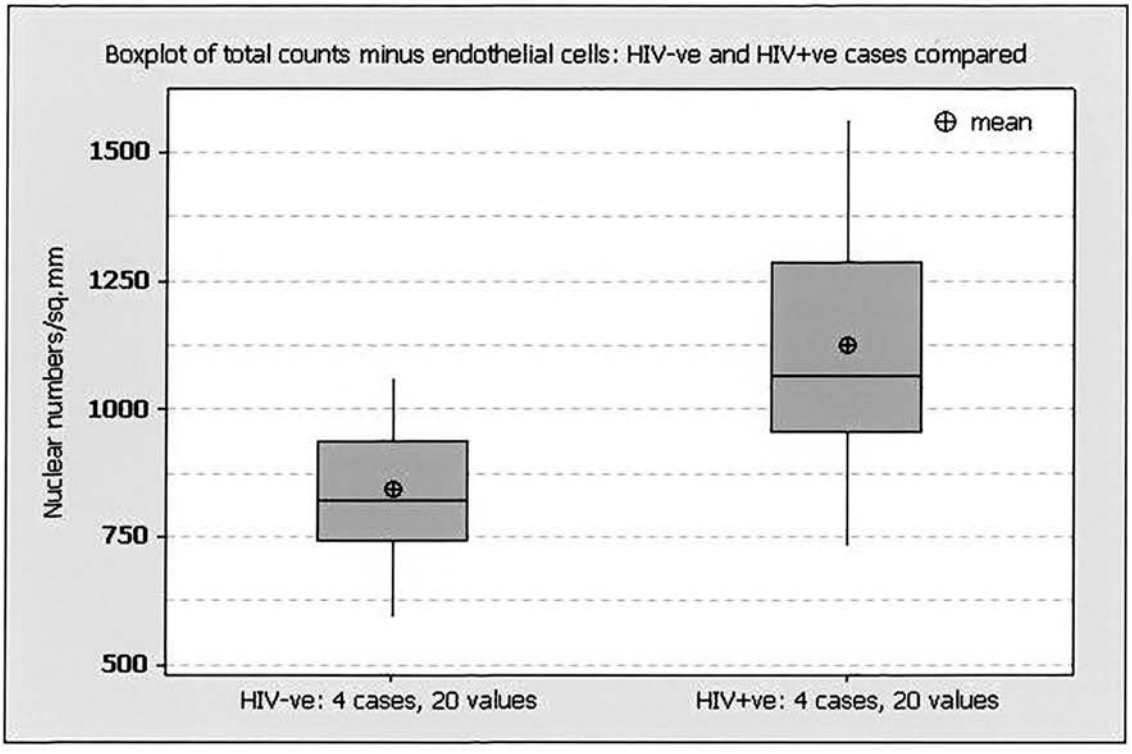
The second set of boxplots (Figures 3.3.13 to 3.3.18) illustrates the significant differences between the collected data from 5 brain areas in the 4 HIV negative (excluding the youngest and oldest HIV negative cases) and 4HIV positive cases, for each of the different cell groups. Each interquartile range box represents the collected data for 20 counts in HIV negative and HIV positive cases. The mean symbols are included in each boxplot.

Fig. 3.3.13



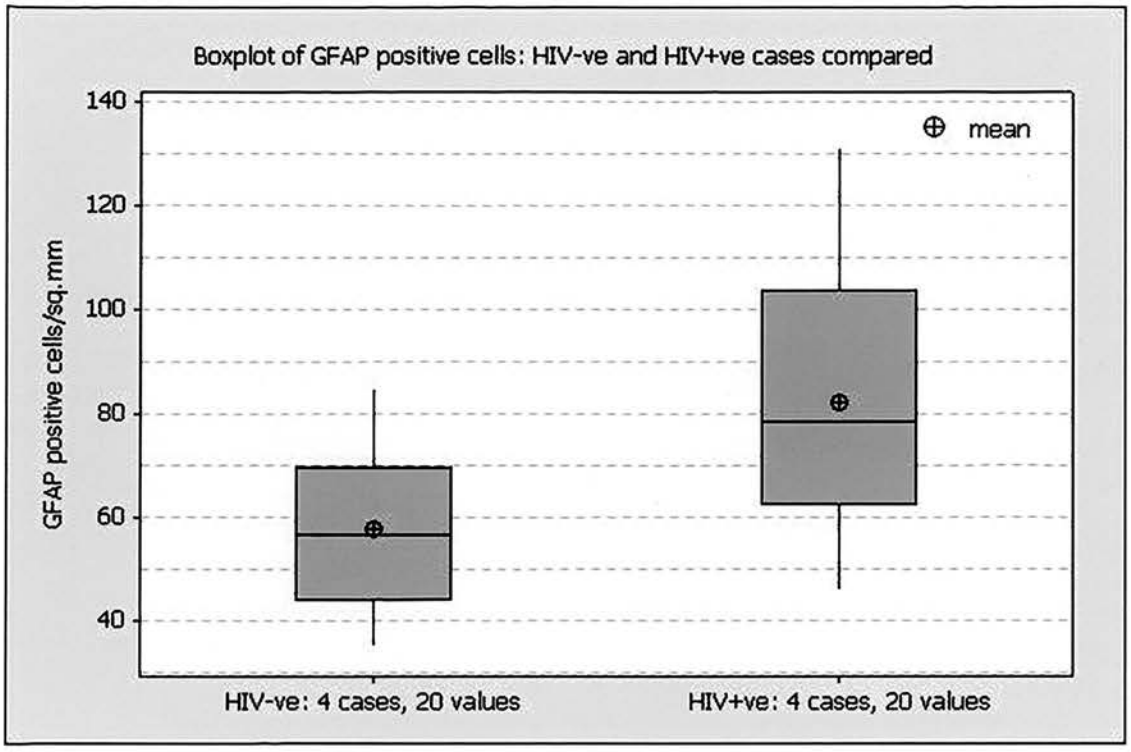
Boxplot of HIV positive and negative cases compared for total nuclear counts – glial and endothelial cells. The difference is significant, p=**0.000**

Fig. 3.3.14



Boxplot of HIV positive and negative cases compared – for glial cell totals only. The difference is significant, p=**0.000**

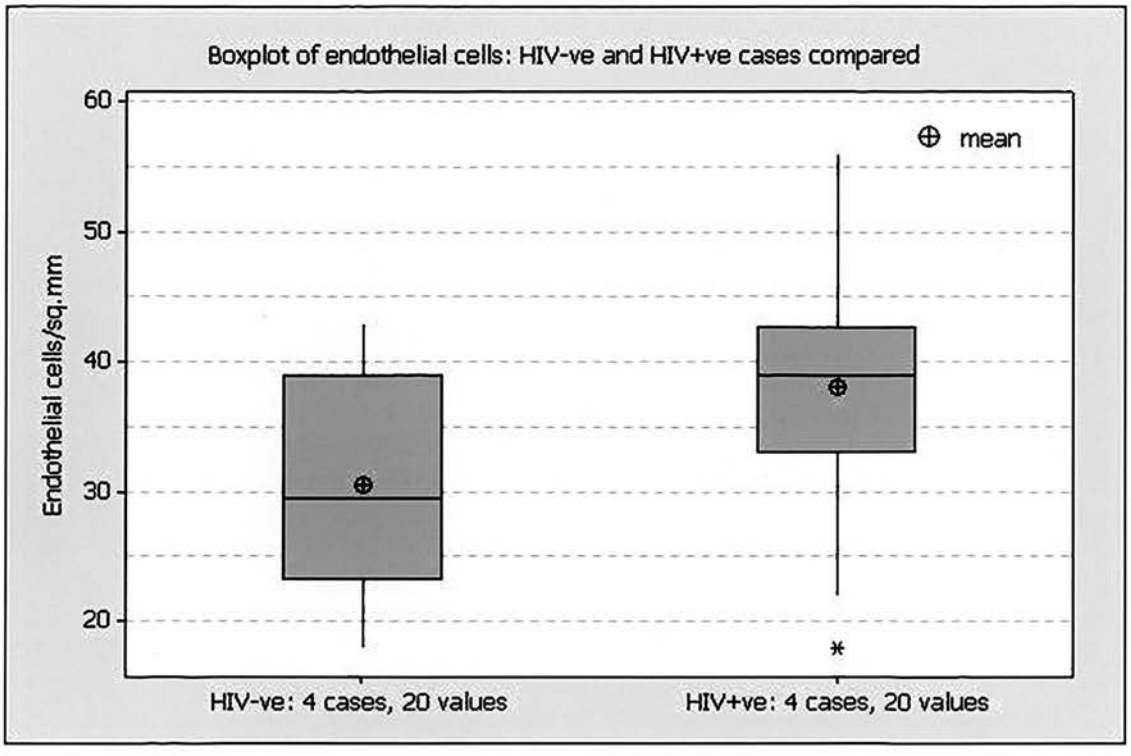
Fig. 3.3.15



Boxplot of HIV positive and negative cases compared – GFAP positive cells.

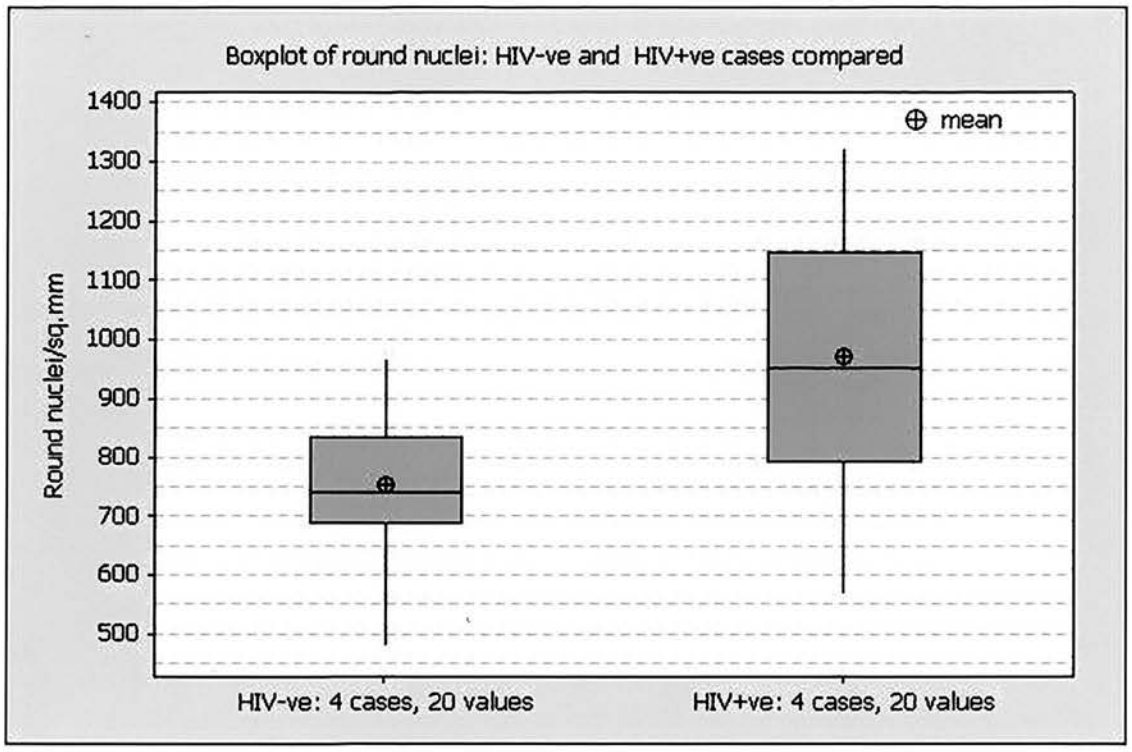
The difference is significant, p=**0.000**

Fig. 3.3.16



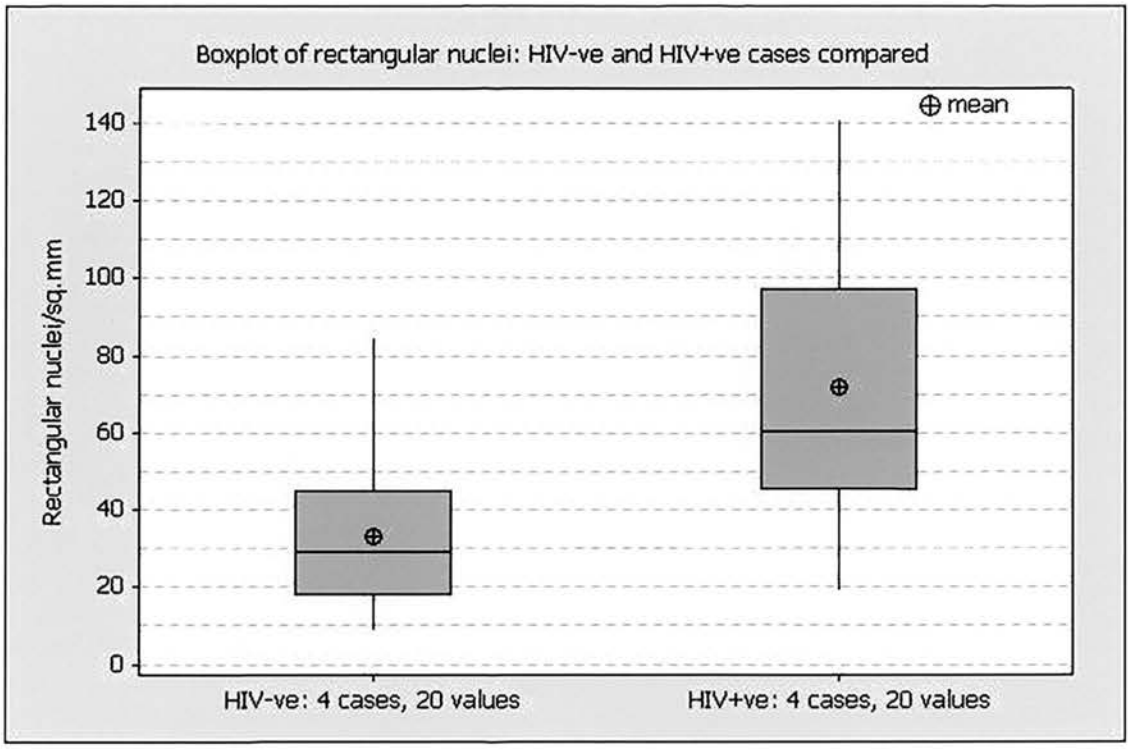
Boxplot of HIV positive and negative cases compared – endothelial cells. The difference is significant, p=**0.007**

Fig. 3.3.17



Boxplot of HIV positive and negative cases compared – round nuclei. The difference is significant, p=**0.000**

Fig. 3.3.18



Boxplot of HIV positive and negative cases compared – rectangular nuclei. The difference is significant, p= **0.000**

The data for the separate groups or categories of counts were analysed by paired t-testing, excluding the youngest and oldest case as explained previously. In each group, and for the HIV negative and HIV positive cases separately, the values of the counts for all areas and for all cases were collected together and compared. As the endothelial cells are clearly not part of the glial cell population there is a case to be made for excluding them from a total

count. On the other hand, they are certainly part of the total nuclear population and so the total counts, including endothelial cells, are also of interest.

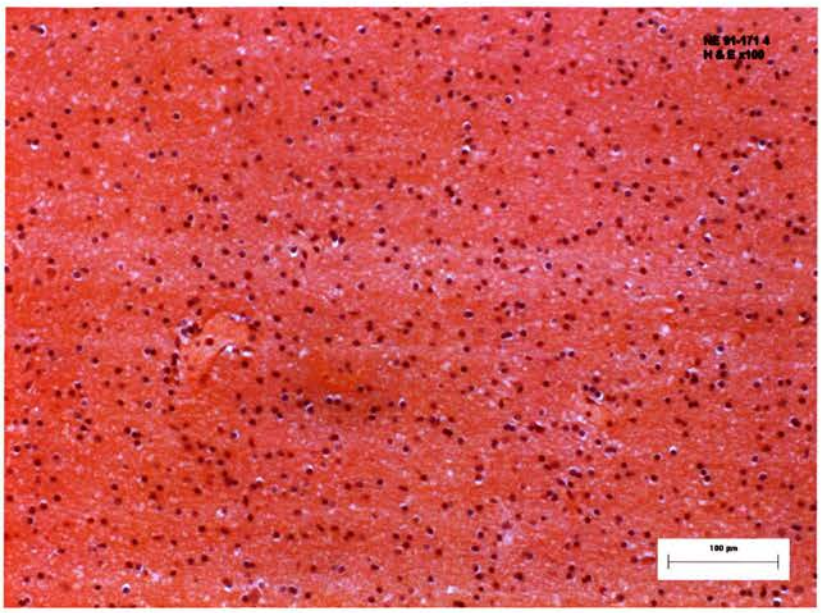
Inspection of Tables 3.3.1 to 3.3.6 confirms the highly significant increases in the counts for each of the nuclear subsets in HIV positive compared with HIV negative cases.

In general, **c80** seems to be the least reactive, and **c16** the most reactive, of the HIV positive cases. These increased counts were found in almost all regions in the HIV positive brains (Appendix 8). The counts for the cerebellum are generally lower than at other levels. In part this may be due to the appearance of marked oedema in the cerebellum. The increase in GFAP positive cells is least marked in **c146**. Case **c80** at cortical level shows least increase in rectangular nuclei. It is of interest that **c31** did not show the maximum increase in any class of cell, despite being the one case with HIVE.

Correlation of Cell Counting with Routine Histology

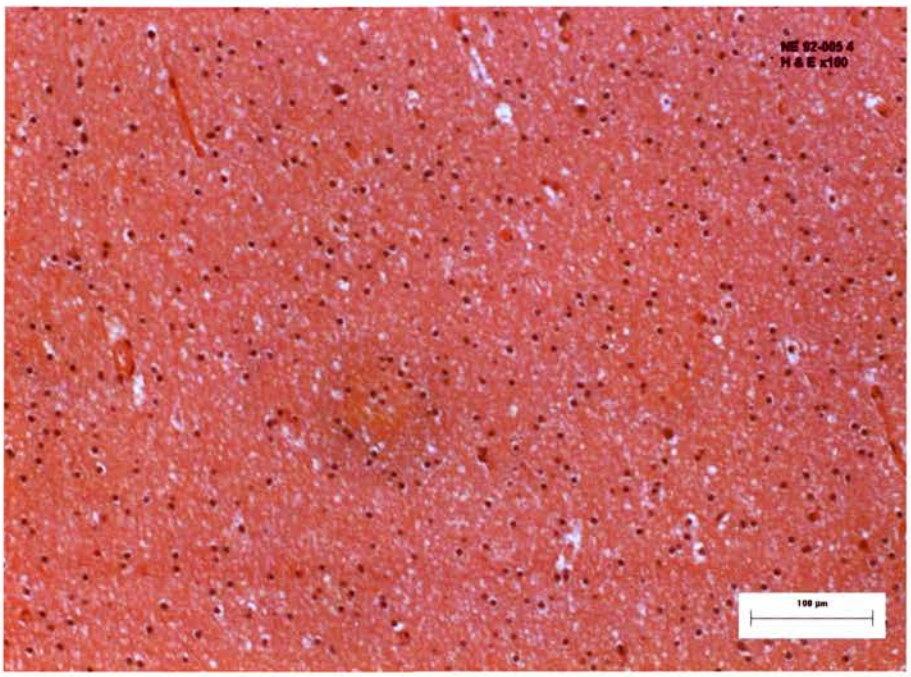
The apparently increased cellularity of the white matter, on simple inspection, was usually confirmed on counting. For example, in an HIV positive case (**c16**), the nuclear numbers appeared increased on inspection of sections of the cerebral convexity (Fig. 3.3.) and this was confirmed with a mean count of 1608 per sq.mm. For comparison, in sections of the cerebral convexity in HIV negative case (**c61**), the white matter nuclear density appeared less (Fig. 3.3.) and the corresponding mean nuclear count was 1063 persq.mm.

Fig. 3.1.19 Cerebral Cortex



Section of the gyral white matter from an HIV positive case (c**16**) showing hypercellularity of the white matter. The total nuclear count was 1,608 per sq.mm. HE x 100

Fig. 3.3.20 Cerebral Cortex



Section of the gyral white matter from an HIV negative case (**c61**) for comparison of cellularity with the HIV positive case shown in Fig. 3.1.19. The total nuclear count was 1,063 per sq.mm. HE x 100.

3.4 - Discussion and Conclusions

The aim of this Initial Study was to attempt to define the glial populations in their totality and, if possible, the contribution of their separate types to the total value in each of various regions of the CNS in this paediatric African cohort. In this size-limited survey there are several findings of interest and which merit further investigation.

1. The study suggests that it is possible to obtain reproducible figures for each glial cell type (defined in the ways indicated above) in specific areas of disease-free brains of HIV negative African children. The question arises as to whether similar values would be found using the same approach if the white matter of Caucasian children, or of adults of differing ethnic origin, were to be investigated.
2. This study also suggests that HIV infection is associated with hyperplasia, i.e. increased numbers, of each of the cell types as defined above. This response appears both diffuse and widespread, occurring as it does in widely different regions of the brain and throughout the neuraxis. In the case of the round nuclei, the objection arises that an influx of lymphocytes may contribute to the total, or that they are rectangular nuclei cut in transverse section, but if so, the pattern of distribution of nuclei appears little different from that of the normal white matter parenchyma. This problem could be explored using immunostaining with lymphocyte markers. The microglial and macrophage populations, with their labile

morphology in pathological conditions, further serve to cloud the issue. It is unclear whether this hyperplasia, which appears to affect all glial populations, is the result of occult HIV infection in the brain, or the result of a common and "remote" stimulus, for example a circulating cytokine.

3. This study suggests that there is also endothelial cell hyperplasia in association with HIV infection. The difference between the means of HIV negative and HIV positive cases is statistically significant. This result was unexpected and the reason for it is not clear. Does it represent a response to a circulating mitotic stimulus? There are possible implications for the integrity of the Blood Brain Barrier and it would be worthwhile exploring this further with specific immunostains, for example, ZO1.

At cellular level, there may be limitations to the usefulness of counts in any particular region. It is known that the density of microglia varies from region to region (Lawson et al. 1990) and also that the numbers of oligodendrocytes vary between different fibre tracts (Friede 1961). The orientation of the fibres probably affects the counts. A cursory check, using an eyepiece graticule, certainly suggests that this is so, the numbers in the obliquely cut internal capsule appearing less than in the curvilinear fibres of the external capsule.

Any enquiry of this sort is beset with difficulties and liable to criticism on several grounds, especially in respect of the identity of the glia, which, even in the experimental animal, can remain elusive. The illuminating results obtained by gold and silver staining in the early days of neurohistology served to intensify the debate, already active throughout Europe, on morphology and function of the neuroglia. The introduction of electron microscopy likewise

provided a further opportunity to examine the glia and their relationships. Immunocytological stains used today vary in their specificity – only a proportion of fibrous astrocytes are stained with the GFAP antibody - and that proportion is affected by physiological and pathological conditions. The other glia, likewise, cannot be visualised exclusively or in their entirety.

Whereas a few decades ago there was a tendency for clinicians and pathologists to confidently assign this sign, or those appearances, to a specific condition, now there is a greater acknowledgment of uncertainty. Current histological texts, when illustrating the neuroglia, tend to suggest that "this cell may be presumed to be an oligodendrocyte" or "that cell is probably an astrocyte".

It is hoped that the pragmatic approach adopted in this study has produced results that stand comparison with any others which refer to glial numbers in human subjects (Esiri et al. 1991; Sjobeck and Englund 2003) It is noteworthy that the proportions of different glial types found in this study are not dissimilar to these previous studies. The limitations of small case number are acknowledged but investigations of this type are very labour intensive and time consuming.

The findings relating to **c85**, a young HIV negative case, are of note in deviating from the general pattern of lower values for nuclear counts in HIV negative cases. Though no specific abnormal findings were detected at the initial screening in 1997 (Bell et al. 1997), these results raise the possibility of significant subtle brain pathology., possibly relating to the history of past

malaria. An alternative explanation is that this case shows less oedema than the others.

Several other limitations in technique and method should be mentioned. At the cutting stage, difficulties were sometimes experienced in obtaining consistent section quality, despite consideration of the usual causes and seeking advice. In view of the unique value of the material, in terms of how it was acquired, as few sections as possible were discarded. Their quality, therefore, may in places appear suboptimal. Another problem arose at the mounting stage – just above the mounting hood the ceiling tiles were starting to crumble, following a leak in a drainage pipe; dust was unavoidably encountered and deposits on some the sections occurred. The difficulty with the immunostaining for lymphocyte and macrophage markers was referred to in the Material and Methods (later overcome in the Main Study).

As mentioned in the results section, many of the cases showed oedema in at least one of the regions examined, especially in the cerebellum, where in addition spaces were sometimes seen, suggestive of nuclear loss, presumably in processing. The impression was that this was more likely to affect the round nuclear population and that it could amount to 10% or more – cerebellar counts may be underestimates of the true number.

As regards the general white matter assessment, the selection of cases aged over the age of 4 years ruled out the potential confounding influences of early stages of myelination. The finding of varying degrees of myelin pallor seemed to be inseparable from the equally frequent finding of early hypoxic neuronal change and oedema. These, in their turn, are a reflection of agonal changes in

children beset with infections and presumed severe metabolic derangements. It seems unwise to draw any firm conclusions about white matter pathology where pallor alone is present. Moreover, assessment of pallor is a very subjective matter and intra-case variability alone precludes useful case comparison. However, the case that showed the most definite pallor, **c31**, also showed conspicuous astrogliosis, especially in the cerebellum, suggesting a morphological basis for the pallor.

Also, in any morphological study, the question of shrinkage of tissue arises and, of course, it is impossible to discount this factor in the cases under study here. However, the post mortem examinations were performed soon after death, at the same locus, and the tissue was processed in a standardised way; and the quality of the histology is consistently high. Any variations in tissue shrinkage are likely to reflect antemortem factors outwith the control of this study.

Chapter 4: The Main Study

4.1 – Introduction

Since microglia are the principal central nervous system (CNS) cell type to be infected with HIV, and viral infections of the brain elicit a lymphocytic response, it is not surprising that most neuropathology studies in AIDS have focussed on inflammatory reactions.

The concept of immune privilege of the CNS – once held to be absolute – is now seen as one in which there is both temporal and spatial latitude (Engelhardt 2006) and the principles involved are essential to the understanding of the neuroinflammatory responses illustrated in this study. Lymphocyte transit into the brain appears to occur at post-capillary venule level rather than in the capillary bed (Raine et al. 1990) and cell passage occurs through the endothelial cell (transcellular route), rather than around it (paracellular route) (Wolburg et al. 2005).

The microvasculature of the grey and white matter, and of the spinal cord, appears uniform at microscopical and ultrastructural level but there are significant differences when compared with the meningeal micro vessels (Allt and Lawrenson 1997). The meningeal, or pial, microvessels have no astrocytic association or envelopment and the endothelia of the cerebrospinal microvessels lack one of the molecular pathways - p-selectin – necessary for neutrophil adhesion (Barkalow et al. 1996). These features help to explain the marked difference in response to inflammation in two intimately associated tissues – the relative impermeability of the CNS parenchyma to

neutrophil access is in strong contrast to the exuberant meningeal response in meningitis.

There is also evidence to suggest that at molecular level there may be subtle differences in pathways involved in inflammatory cell access between the cerebral and spinal microvasculature. A technique known as intravital microscopy (Piccio et al. 2002) has revealed in mice that activated lymphocytes may roll on endothelium prior to transit across walls of microvessels in the brain, but are captured immediately in spinal cord microvessels. This suggests a regional specialization which might help to explain differing localization and intensity of the inflammatory process within the CNS. Furthermore, it was shown that priming of the endothelium of microvessels by an injection of lipopolysacharride or TNFα could enhance the subsequent sequestration of activated lymphocytes (Piccio et al. 2002) and thereby raises the possibility of CNS involvement by systemic stimuli(Engelhardt 2006).

In health there is limited immune surveillance in the the subarachnoid and perivascular spaces . The lymphocyte population of the cerebrospinal (CSF) is replenished twice daily by interchange with the bloodstream through the choroid plexus. As noted in Chapter 1, T and B lymphocytes enter the normal CNS in small numbers (Hickey 1999; Anthony et al. 2005). In disease states lymphocyte interactions with the CNS are much enhanced.

Much of what is known of the cellular and molecular biology of inflammatory cell dynamics in CNS inflammation is derived from the study of an animal model of demyelination known as experimental allergic encephalitis (EAE), first described in 1935(Rivers 1935). In EAE, rodents are sensitized against a component of myelin and the effects are studied under varying conditions. Much of the experimental work

has focused on the dynamics of CD4 T-cells (Ben-Nun et al. 1981; Beraud et al. 1993) but more recently the pathogenic role of CD8 T-cells has been explored (Huseby et al. 2001) (Ji and Goverman 2007).

Infiltrates of lymphocytes and macrophages are a characteristic feature of acute and chronic inflammatory diseases of the CNS – e.g. viral infections and multiple sclerosis, and in CNS ischaemia and trauma. The degree and location of the infiltrates vary according to the nature of the insult; there may be no more than a few cells along the course of small vessels or there may be multilayered vascular cuffing with focal and diffuse parenchymal cellular collections. The recruitment of such cells is complex and there are subtle interdependencies between the cells of the innate and adaptive immune system. However the initial part of the process – that of leucocyte adhesion and passage through the endothelium, known as transmigration – is well defined in molecular biological terms (Muller 2002). It is governed by the interaction, by means of chemical mediators, of adhesion molecules on the surfaces of leucocytes and endothelial cells. The most important receptors belong to three main groups, each having a slightly different profile of activity:-

The selectins are named because of their affinity with sugar binding mammalian lectins. The three main selectins are E- selectin found in endothelium, P-selectin in endothelium and platelets and L-selectin, present on most leucocytes. The selectins are mostly involved in rolling and adhesion of leucocytes on the endothelial surface.

The immunoglobulin family contains two well recognised adhesion molecules; intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) These are present on endothelial cells. ICAM-1 is involved in transmigration.

The integrins are a large family of transmembrane proteins present on many cell types and which bind to endothelial cells or to other cell types through ICAM-1 or VCAM-1. Induction, or up-regulation, of adhesion molecules on endothelia occurs in response to a variety of stimuli ranging from local trauma to circulating cytokines. In the particular case of CNS endothelia, circulating cytokines such as TNF-α, locally produced cytokines (by perivascular cells) including TNF-α and IL-1, and cell to cell signalling originating in the end feet of activated microglia and astrocytes, probably all play a part (Dorries 2001). Furthermore, in HIV infection, endothelial cells secrete immuno-active molecules in response to certain HIV products, such as gp120 and Tat (Banks et al. 2006).

The behaviour of lymphocytes which have traversed the blood brain barrier (BBB) is subject to a number of chemotactic stimuli, at least one of which is determined by activated macrophages. A study on EAE in mice showed that whilst macrophage depletion allowed transvascular passage of autoreactive T-cells, parenchymal infiltration did not follow and demyelination did not occur (Tran et al. 1998). The influx of CD8 T-cells into the CNS in viral infection is partly determined by signalling from natural killer cells which precede them as an innate immune response. Natural killer cells secrete IFN-γ, which is a major activator of CD8 T-cells (Dorries 2001). Activated microglia acquire antigen presenting properties and in their interaction with CD8 T-cells confer cytopathic ability. Another pathway is for antigen specific CD8 T-cells to gain independent access through interaction with MHC class 1 expressing endothelial cells (Galea et al. 2007).

T-cells and HIV infection

One of the features of early HIV infection is the presence of perivascular and leptomeningeal lymphocytic infiltration, which has been interpreted as evidence of an immune response to the presence of HIV (Bell et al. 1993) (Gray et al. 1992). A few early studies on the brain lymphocytic population in AIDS failed to detect significant numbers in the perivascular position and could not find a difference between AIDS brains and controls (Tyor et al. 1992) (Wesselingh et al. 1993). However later studies did show perivascular lymphocytic aggregations both in adults (Weidenheim et al. 1993) and in children in AIDS (Katsetos et al. 1999). A more recent study (Petito et al. 2003) investigated the dynamics of lymphocytic infiltration in the hippocampus of AIDS subjects and showed that perivascular T-lymphocytes were increased in the peri-hippocampal white matter in the presence of HIV encephalitis (HIVE) but were rare or absent in controls and AIDS cases without HIVE. These T-lymphocytes included CD45RO+ (activated/memory cells) and CD3+, CD4+ and CD8 cells. Parenchymal CD45RO+ and CD3+ lymphocytes were numerous in areas adjacent to HIVE foci. Minimal evidence of infection of lymphocytes was detected by HIV p24 antigen testing. In addition, all lymphocyte subsets were seen to participate in the formation of microglial nodules along with microglia, macrophages and multinucleate giant cells. Several reasons for the accumulation of lymphocytes were considered in this study, including factors favouring transmigration and cytokine induced chemotaxis. Another possible mechanism involving reduced apoptosis of infiltrating cells, due to astrocyte malfunction, has also been proposed (Gold et al. 1996). The proximity of lymphocytes to neurons in HIVE cases may suggest a neurotoxic relationship (Petito

et al. 2006). Another possible adverse effect was enhanced productive HIV infection through the agency of secreted T-cell factors (Tornatore et al. 1991).

A further study by the same group (Petito et al. 2006) explored the CD8 and cytotoxic lymphocyte populations in HIVE. In this study, the CD8 sub-population of activated or cytotoxic T-lymphocytes was identified by the possession of granzyme or perforin granules, detected by immunocytochemistry. The areas studied were the hippocampus and basal ganglia and the subjects were a small group AIDS patients – some with HIVE and some without. Perivascular CD8 cells were more numerous in HIVE and in AIDS cases without HIVE than in controls. CD8 parenchymal cells were frequent in the HIVE cases and were largely absent from the AIDS cases lacking HIVE, and from controls. These findings were paralleled by those for those CD8 granule positive cells, except that the numbers were smaller. As in the previous study, lymphocytes were found in close contact with neurons in HIVE cases and the authors proposed that their presence in such a situation could serve as a marker for productive infection, if lymphoma and opportunistic infection were excluded. Another inference was that glial cells were also susceptible to the attention of cytotoxic T-lymphocytes, though this activity was not specifically detected.

McCrossan et al (McCrossan et al. 2006) examined lymphocytic infiltrates and inflammatory change in 15 presymptomatic HIV cases, in addition to CNS viral genetic analysis. The subjects had died of causes unrelated to HIV and no evidence of HIVE or HIV associated neuropathology was found at post mortem; none had received anti-retroviral therapy. Perivascular CD8 lymphocytes were found in small numbers in all HIV cases and 3 cases showed large numbers of perivascular cells,

extending to the parenchyma in one of these cases. A correlation was shown between the degree of CD8 infiltration and both viral load and markers of immune cellular activation – CD14, CD16 and CD68.

HIV-1 specific cytotoxic T-cells are found in the blood of HIV positive patients and are thought to be responsible for viral control until the late stages of the disease (Brander and Riviere 2002). In children, a falling blood CD8 T-lymphocyte count and a rising monocyte count were found to be predictors of HIV-1 progressive encephalopathy (Sanchez-Ramon et al. 2003).

Another aspect of CD8 immunopathology, mentioned briefly in Chapter 1, is the immune reconstitution syndrome (IRIS), in which HIV infected patients on HAART suffer severe relapse of their disease. The most significant CNS post mortem finding in this condition is perivascular and parenchymal accumulation of large numbers of CD8 T-lymphocytes (Miller et al. 2004). This is thought to be due to the abrupt recovery of a severely depleted peripheral CD8 lymphocyte population and its subsequent influx into a relatively unprotected CNS (Berkeley et al. 2008).

B lymphocytes and HIV infection

Very little was known about CD20 lymphocytes (B-lymphocytes) in the brain until 2003, when a seminal paper was published, documenting not only the discovery of B-lymphocytes in normal brain but also their presence in several groups of HIV positive subjects (Anthony et al. 2003). In the normal adult brain, B-lymphocytes were found in very low numbers in all regions of the brain and were truly parenchymal in location. These cells possessed an activated phenotype, expressing CD79a. In asymptomatic HIV subjects compared with controls, they were found in

increased numbers in both the perivascular and parenchymal compartments. Increased numbers of perivascular T-cells in a similar group of subjects had been noted in previous studies (Bell et al. 1993) (Tomlinson et al. 1999). In some AIDS subjects in whom blood CD4 and CD8 lymphocyte counts had fallen to low levels, a decline in perivascular and parenchymal B lymphocytes to negligible proportions was noted. This finding was in accordance with a similar scarcity of T lymphocytes noted in AIDS cases (Bell et al. 1993) (Bell et al. 1996). In contrast, a subset of AIDS subjects was found to have marked pleomorphic lymphocytic collections in the perivascular compartment. In these loci B cell numbers were increased. The significance of these aggregations was uncertain – it was not thought to be a pre-lymphomatous condition since the distribution was diffuse rather than focal, and in primary CNS lymphoma (PCNSL) such collections are uncommon away from lymphomatous foci. Furthermore, the B cells in such collections were Epstein-Barr virus (EBV) negative. The authors also felt that these pleomorphic collections were unlikely to be an extension of the perivascular cells seen in asymptomatic cases, but rather represented a separate process.

Microglia/macrophages and HIV infection

The general principles of entry for immune cells into the CNS are considered in Chapter 1, and additional factors probably operate in respect of monocytes. In late HIV infection, the increased numbers of monocytes in the circulation (cf. decreased numbers of lymphocytes at this stage) favours increased migration of monocytes to the brain (Pulliam et al. 1997).

The essential features of microglial micro-anatomy and physiology and their role in the pathogenesis of HIV infection were considered in Chapter 1. As regards their proportion in the human brain, various figures are quoted, such as 12% of the total, (Sedgwick et al. 1991), 15% in some parts of the CNS (Hauwel et al. 2005) and 5-10% in HIVE (Fischer-Smith et al. 2004). Microglia are the resident immune cells of the brain and have a very low turnover in health. They exist in a resting state as cells with a ramified morphology and there is no overlap of individual microglial territories. In the absence of stimulation of any kind, their resting state is regulated by astrocytic secretion of a molecule known as macrophage stimulating factor (M-CSF) (Eder and Fischer 1997). Neurons also suppress activation of microglia through the action of neurotrophins. Interaction with T-cells may result in marked reciprocal activation, depending on the circumstances (Benveniste et al. 2004). Quiescent microglia express immune receptors and ligands at very low levels. A variety of stimuli can cause activation and these include trauma, bacterial lipopolysaccharide (LPS), DNA and viruses (Lokensgard et al. 2000). A change occurs to a more rounded phenotype, with reduced processes, and surface receptors, including Toll-like receptors (TLRs) are upregulated. TLRs are a family of receptors akin to the Toll receptors of the much studied fruit fly *Drosophila,* which recognize molecules associated with bacterial pathogens. These in turn trigger the secretion of cytokines such as TNFα, and interleukins 6 and 10 (Bsibsi et al. 2002)

Other important secretory products of microglia include the matrix metalloproteinases (MMPs). These are a family of proteolytic enzymes associated with the extracellular matrix. Activation results in damage both directly, and also

following the conversion of TNF precursor into active TNF. The MMPs are implicated in BBB breakdown and tissue destruction in HIV (Ghorpade et al. 2001).

In addition to the deleterious effects of microglial activation, it should be noted that there is also a protective role for microglia (Gras et al. 2006) through the glutamate cycle though the dynamics of this process are not yet fully understood.

In activation, a complex and dynamic situation exists in which the whole spectrum of microglial function may be displayed including antigen presentation, pro- and anti- inflammatory activity, migration and proliferation (Benveniste 1998). In vitro studies have shown that microglia can, through auto–secretion of molecules such as IL-1 β and IL-4, stimulate self proliferation. In HIV infection, whether or not a microglial cell is infected, activation may occur. Early infection of microglia is believed to occur and since microglia are long lived, the virus can remain in a latent state for many years, suppressed by both systemic (Sadagopal et al. 2008) and local innate immune control (Sopper et al. 1998). These controls eventually fail, probably because of falling CD8 counts and rising monocyte levels in the blood, and local factors, which may include positive feed-back loops (Wilt et al. 1995). In the increasingly rich cytokine CNS microenvironment, latent virus is awakened. TGFβ has been shown to change the viral long terminal repeats (LTR) in astrocytes (Coyle-Rink et al. 2002) and IL-4 and IL-10 can cause increased viral replication in microglia (Wang et al. 2002).

The formation in HIVE of the pathognomic multinucleate giant cells is believed to arise from the fusion of infected microglia (Michaels et al. 1988). Microglial nodules, also a feature of HIVE, should perhaps be termed inflammatory nodules (Vintners 2008) as they contain other cell types, including CD8 lymphocytes.

The biology of the monocyte/macrophage/microglial populations has been of crucial importance in the understanding of neuroAIDS from the outset of the epidemic. Interest was focussed initially on the function of the infected monocyte/macrophage as a vehicle for transport, and then as a reservoir of infection, sequestered from the normal surveillance of the immune system. In recent years the emphasis has been on the relationship between activated and infected CNS macrophages and microglia, their production of toxic cytokines and viral products, and parenchymal damage. As HIV positive subjects survive longer on HAART, and neurodegenerative conditions such as Alzheimer's disease become superimposed, there is a need to explore the relationship on economic grounds as well as those of science.

Confusion exists regarding the terminology of macrophage populations and disagreement as to which marker detects which cell type, and whether or not activation is present (Williams et al. 2001; Williams et al. 2001; Fischer-Smith et al. 2004). There seems to be a consensus that CD68 detects all activated cells of monocyte/macrophage lineage, including microglia (a fine point being when does an activated microglial cell become a macrophage?). CD68 may not detect resting microglia, since the markers CD16 and MHC class II may occasionally detect higher levels in AIDS subjects lacking HIVE (Anthony et al. 2005).

CD14 is generally accepted as a marker for perivascular macrophages. The term perivascular cell could apply equally to macrophages and microglia as both occur in a perivascular position. The microglia in such a locus share with astrocytes a small percentage (5-13%) of the surface area of the BBB, by means of end feet (Lassmann and Hickey 1993). These cells should be termed parenchymal microglia according to

one author (Williams et al. 2001). However parenchymal microglia can also express low levels of CD14, both under in-vitro and in-vivo conditions (Becher and Antel 1996).

The situation regarding CD16 expression is no less complex. Perivascular and parenchymal cells that are CD16 positive are probably a heterogenous populaton derived from monocytes recently recruited from the blood, activated and sometimes infected perivascular macrophages, and activated parenchymal microglia (Fischer-Smith et al. 2004).

As regards the microglial cell type which is principally infected, both at initial infection and in the late stages of AIDS, opinion differs. In a very carefully controlled study of simian immunodeficiency virus (SIV) there seemed little doubt that the perivascular macrophage population, rather than the parenchymal microglia, was the major repository of HIV infection (Williams et al. 2001). This finding was supported by a study of HIVE cases in which HIV p24 immunostaining was located in CD14 positive cells in both perivascular and parenchymal sites (Fischer-Smith et al. 2001). However Cosenza (Cosenza et al. 2002) considered that the majority of productively infected cells were parenchymal microglia.

The CNS parenchymal changes in HIV/AIDS in children

Naturally the opportunities to study the pathological changes in the CNS of pre-symptomatic individuals are limited and the few published studies are of small numbers of subjects, usually drug dependent individuals (Gray 1997), and occasional single case studies (Lenhardt et al. 1988). In 4 drug dependent pre-symptomatic cases (Budka 1991), compared with a similar group of HIV negative controls,

conspicuous differences included the much greater degree and extent of vascular inflammation, myelin pallor and astrocytic gliosis in the HIV positive group. Perivascular cuffing was a consistent finding, associated with small parenchymal or leptomeningeal vessels, especially veins. Nothing is known regarding the neuropathology of the early stages of HIV infection in children.

Regarding the symptomatic stages of AIDS, much of our knowledge of the changes in late HIV infection stems from post-mortem studies conducted in the USA before the availability of highly active anti-retroviral therapy (HAART). The first study to document the CNS appearances in children with AIDS (Epstein et al. 1985) provided the clinical details of four children aged from 9 months to 11 years, who had developed neurological illnesses, associated with immunodeficiency. Three of these children died and cerebral atrophy was found in all. Microscopical examination showed the presence of multiple foci of microglial cells, present throughout the brain and particularly in the basal ganglia, thalamus and brain stem. In one case these nodules had necrotic centres. Gliosis was prominent in one case, which also showed marked neuronal loss. Cytomegalovirus (CMV) was present in another.

A large post-mortem study of 268 AIDS patients in 1991(Kure et al. 1991) included 31 infants and children. Sub-acute AIDS encephalitis was found in 26% of adults and in 48% of paediatric brains. HIV antigen gp41 testing in a sample of 100 adults and 20 paediatric brains was positive for 78% of the former and 40% of the latter. Opportunistic infections were uncommon in paediatric brains compared with adults and were restricted to 2 cases of CMV encephalitis. Brain weight was generally reduced and in some cases this reduction was marked, and associated with extensive gliosis in the white matter. Microcalcification in the basal ganglia was the

most frequent finding and in about a third of the children, microglial nodules and multinucleated cells were present. Myelination was frequently defective due either to delay, or to myelin loss, which appeared multifocal. In those spinal cords that were examined, myelin was reduced in the corticospinal tracts. Six of the 31 paediatric brains showed the presence of lymphomas and all were B-cell tumours.

A difference was noticed between younger and older children in respect of HIV antigen detection and inflammation, both of which were commoner in older children. The authors surmised that this might be related to factors such as lower TNF levels and relative immaturity of the microglial population in younger children.

A further large study of paediatric HIV neuropathology, in the period before HAART, was published in 1993 (Dickson et al. 1993). The findings of 45 autopsies were recorded, for brain and spinal cord in 18 cases, for the brain only in 24 cases and the spinal cord only in 3 cases. All cases were symptomatic. Reduced brain weight with atrophy was a common finding. Gliosis with basal ganglia microcalcification was also common. HIVE, diagnosed on the basis of microglial nodules and multinucleate cells, was judged to be present in just over 60% of brains. As noted in the previous study, a trend towards greater levels of inflammation in older children was detected. Cerebrovascular pathology was found in 22 (52%) of cases, chiefly in the form of anoxic-ischaemic encephalopathy. Lymphomatous deposits were found in 8 brains – the youngest being only six months of age. Opportunistic infection was uncommon and only CMV was found.

The next large study to be published was on HIV/AIDS in children in Africa, namely the 1997 study of Bell et al (Bell et al. 1997), from which the present study derives. Following the publication of the general pathology and baseline

neuropathology findings, it was clear that this cohort warranted further investigation with a wider immunohistochemical panel, to determine both the CNS inflammatory infiltrates associated with HIV infection and the associated white matter reactions. The following **hypothesis** was formulated:

The HIV positive children would show a higher level of activated microglia and infiltrating lymphocytes than that shown by the HIV negative children, despite the high level of background brain pathology in the latter group.

Accordingly, in this Main Study, the **aim** was to quantify and compare microglia/macrophages (innate neuroimmune system) and lymphocytes (adaptive neuroimmune response) in selected HIV positive and negative children and to review the associated changes in axons and astrocytes.

4.2 - Materials and Methods

In this study two different parts of the brain, the basal ganglia and the hippocampus, were examined in an attempt to define the degree and extent of inflammation in HIV negative and positive children; and both grey and white matter were examined to try to detect any differential involvement of these parts in neuroinflammation. Because of differing block availability for these two brain areas, the cases used differ very slightly, (Group A1, basal ganglia blocks, n=40 and Group A2, hippocampus, n=40), but the cases were age matched. Groups A1 and A2 were each made up of 20 HIV negative and 20 positive cases. A battery of immunostains

was applied, as described in Chapter 2. The sections were then assessed in several ways. The haematoxylin and eosin sections, and those stained by Luxol fast blue, were examined by simple microscopy with a view to identifying the main pathological features and degree of myelin pallor, if present. The degree of microglial and macrophage activation (area of staining-sq.μm/4 sq.mm of section) was determined by image analysis for CD16 and CD68. The assessment of CD14 staining was by simple grading partly because of potential confounding CD14 positivity in intravascular cells (less of a problem with CD68 and not a problem with CD16) and partly, the diffuse pattern of CD14 staining precluded precise cell counts. The presence and distribution of lymphocytes was assessed by simple counting of CD8 and CD20 positive cells. In contrast to microglia, the defined cell outlines of lymphocytes facilitated simple counting.

Astrocyte activation was assessed and graded simply according to the intensity and pattern of distribution of glial fibrillary acidic protein (GFAP) positivity. Axonal pathology was detected by β amyloid precursor protein (βAPP) immunostaining and recorded as to its presence and extent.

The results for all stains were tabulated, displayed and analysed using the statistical software, Minitab.

The 40 cases for the main study, chosen from the original cohort, did not include the 10 initial study cases. Children aged 11 and 12 years were also excluded from the main study because they were much older than the majority of cases in the original cohort. **Group A1**, consisting of basal ganglia blocks, often referred to the Tables as "BG", was selected in the following way: seven HIV negative and seven positive cases, each set drawn from the youngest and middle age groups in the cohort, while

six HIV negative and six HIV positive cases were chosen from the oldest age group (n=40). These cases are listed in Table 4.2.1, in which the cases are sorted by age. This table also contains the set of the hippocampal blocks (**Group A2**), which differs in composition slightly because of block availability. Table 4.2.1 contains 48 cases and there are 32 cases in common between Groups A1 and A2. The pathology findings, available from the results of routine staining as described in the original study, are also listed. Tables 4.2.2 and 4.2.3 show the basal ganglia set (Group A1) and hippocampus set (Group A2) respectively, sorted on HIV status, together with general neuropathology findings. In Tables 4.2.2 and 4.2.3, the cases are listed in order of acquisition to the original study, and no longer by age. This is the order in which cell quantitation results are provided in later Tables in the Results.

From both Groups A1 and Group A2, a subset of 5 HIV negative and 5 HIV positive cases was selected on the basis of having no significant neuropathological findings. The case numbers for these subsets are shown in Tables 4.2.2 and 4.2.3. This grouping allowed comparison between HIV negative and HIV positive cases without the very diverse and major CNS pathology present in most of the other cases. These subsets appear in Figures and Tables and are designated **Groups B1 and B2** (Tables 4.2.2 and 4.2.3). Sampling of grey matter for histological assessment and quantitation was from the hippocampal and parahippocampal gyrus, and of white matter from as widely-spaced areas as possible – the total area sampled being 4 sq. mm. for each case.

Table 4.2.1 Joint basal ganglia and hippocampus set sorted on age

Case No	Age*	Sex	HIV	BG♦	Hippo♣	CNS pathology findings on routine staining
c82	0.2	F	+	Y	Y	cerebellar infarct & microhaemorrhages
C157	0.2	M	+	Y	Y	purulent meningitis
C77	0.3	F	-	Y	Y	possible septicaemia
C54	0.4	M	-	Y	Y	meningoencephalitis & infarcts
C110	0.4	M	+	Y	Y	toxoplasmosis
C113	0.4	M	-	Y	N	no significant findings
C129	0.4	F	+	Y	Y	low grade lymphocytic infiltrate
C131	0.4	M	+	Y	Y	low grade encephalitis
C150	0.4	F	+	Y	Y	nothing significant
C52	0.5	M	-	N	Y	no significant findings
C89	0.5	F	-	Y	Y	purulent meningoencephalitis
C94	0.5	M	-	Y	Y	no significant findings
C100	0.5	F	-	Y	Y	no significant findings
C109	0.5	F	-	Y	Y	purulent meningitis
C23	1.5	M	-	N	y	no significant findings
C60	1.5	F	-	Y	Y	no significant findings
C74	1.5	F	-	Y	N	no significant findings
C93	1.5	F	-	Y	Y	no significant findings
C125	1.5	M	+	Y	Y	no significant findings
C126	1.5	F	+	Y	Y	no significant findings
C33	1.6	F	-	Y	Y	possible septicaemia
C50	1.6	F	-	Y	Y	no significant findings
C59	1.6	M	-	Y	Y	purulent meningitis
C76	1.6	M	-	Y	N	no significant findings
C91	1.6	M	+	Y	Y	low grade lymphocytic infiltrate

C121	1.6	M	+	Y	N	cerebral oedema
C5	1.7	F	+	Y	Y	no significant findings
C55	1.7	M	+	Y	Y	low grade lymphocytic infiltrate
C51	1.8	F	+	N	Y	low grade lymphocytic infiltrate
C86	1.8	F	-	N	Y	no significant findings
C128	1.8	F	+	Y	N	low grade lymphocytic infiltrate
C70	2.1	F	+	N	Y	low grade lymphocytic infiltrate
C63	2.5	F	-	N	Y	no significant findings
C117	2.9	M	-	Y	Y	cerebral malaria
C122	3.3	F	+	Y	Y	low grade lymphocytic infiltrate
C8	3.5	F	+	Y	Y	subtle widespread wm damage
C62	4.0	F	+	Y	Y	low grade lymphocytic infiltrate
C67	4.0	F	-	Y	N	no significant findings
C101	4.0	M	-	Y	Y	possible septicaemia
C140	4.0	M	+	Y	Y	no significant findings
C24	5.0	M	+	Y	Y	measles encephalitis
C42	5.0	F	-	Y	N	no significant findings
C57	5.0	F	-	Y	Y	no significant findings
C124	5.0	M	-	N	Y	no significant findings
C9	5.1	F	+	Y	Y	medullitis
C16	5.3	F	+	N	Y	no significant findings
C104	6.0	M	-	Y	Y	cerebral malaria
C152	6.4	M	+	Y	N	HIV encephalitis & toxoplasmosis

Notes for Table 4.2.1

1. The basal ganglia and hippocampus sets differ slightly in composition due to varying block availability.

2. * The ages were originally recorded in this decimal format as estimates derived from somatic length. The limitations of this approach are considered in the text and the format is retained here to conserve data integrity.

3. BG♦ refers to blocks selected as Group A1. Hippo♣ refers to blocks selected as Group A2.

Table 4.2.2 - Basal ganglia cases (Group A1) sorted on HIV status and showing the pathology findings and causes of death. The Group B1 subset cases are entered below the main HIV sets

Code	Age	Sex	HIV	CNS pathology findings on routine Staining	Causes of death
HIV negative cases					
c33	1.6	F	-	possible septicaemia	pulmonary infarction
c42	5.0	F	-	no significant findings	pneumonia
c50	1.6	F	-	no significant findings	pneumonia malaria
c54	0.4	M	-	meningoencephalitis and infarcts	no significant findings
c57	5.0	F	-	no significant findings	lymphoma - B-cell
c59	1.6	M	-	purulent meningitis	malnutrition
c60	1.5	F	-	no significant findings	malaria pulmonary infarction
c67	4.0	F	-	no significant findings	pneumonia
c74	1.5	F	-	no significant findings	pneumonia
c76	1.6	M	-	no significant findings	colitis
c77	0.3	F	-	possible septicaemia	sickle cell crisis

c89	0.5	F	-	purulent meningoencephalitis	pulmonary abscess
c93	1.5	F	-	no significant findings	colitis
c94	0.5	M	-	no significant findings	no significant findings
c100	0.5	F	-	no significant findings	pneumonia - measles
c101	4.0	M	-	possible septicaemia	septicaemia
c104	6.0	M	-	cerebral malaria	malaria
c109	0.5	F	-	purulent meningitis	pneumonia
c113	0.4	M	-	no significant findings	pneumonia
c117	2.9	M	-	cerebral malaria	malaria
HIV negative cases for **Group B1** - c50,c67,c76,c93 and c113					
HIV positive cases					
c5	1.7	F	+	no significant findings	CMV enteritis and pneumonia
c8	3.5	F	+	subtle widespread wm damage	pyothorax post measles
c9	5.1	F	+	medullitis	disseminated herpes
c24	5.0	M	+	measles encephalitis	pneumonia CMV diseminated
c55	1.7	M	+	low grade lymphocytic infiltrate	pneumonia strongyloidosis
c62	4.0	F	+	low grade lymphocytic infiltrate	pneumonia
c82	0.2	F	+	cerebellar infarct & microhaemorrhages	pneumonia
c91	1.6	M	+	low grade lymphocytic infiltrate	pneumonia strongyloidosis
c110	0.4	M	+	toxoplasmosis	pneumonia measles
c121	1.6	M	+	cerebral oedema	pneumonia
c122	3.3	F	+	low grade lymphocytic infiltrate	pneumonia
c125	1.5	M	+	no significant findings	pneumonia
c126	1.5	F	+	no significant findings	TB - miliary
c128	1.8	F	+	low grade lymphocytic infiltrate	pneumonia
c129	0.4	F	+	low grade lymphocytic infiltrate	pneumonia
c131	0.4	M	+	low grade encephalitis	CMV enteritis
c140	4.0	M	+	no significant findings	pneumonia

c150	0.4	F	+	no significant findings	pneumonia
c152	6.4	M	+	HIV encephalitis & toxoplasmosis	pneumonia, interstitial
c157	0.2	M	+	purulent meningitis	pneumocystis pneumonia CMV
HIV positive cases for **Group B1** - c5, c125,c126,c140 and c150					

Table 4.2.3 - Hippocampus cases (Group A2) sorted on HIV status and showing the pathology findings and causes of death. The Group B2 subset cases are entered below the main HIV sets

Code	Age	Sex	HIV	CNS pathology findings on routine staining	Causes of death
c23	1.5	M	-	no significant findings	enteritis pulmonary CMV
c33	1.6	F	-	possible septicaemia	pulmonary infarction
c50	1.6	F	-	no significant findings	pneumonia malaria
c52	0.5	M	-	no significant findings	pneumonia
c54	0.4	M	-	meningoencephalitis & infarcts	no significant findings
c57	5.0	F	-	no significant findings	lymphoma - B-cell
c59	1.6	M	-	purulent meningitis	malnutrition
c60	1.5	F	-	no significant findings	malaria pulmonary infarction
c63	2.5	F	-	no significant findings	pneumonia colitis
c77	0.3	F	-	possible septicaemia	sickle cell crisis
c86	1.8	F	-	no significant findings	pneumonia
c89	0.5	F	-	purulent meningoencephalitis	pulmonary abscess
c93	1.5	F	-	no significant findings	colitis
c94	0.5	M	-	no significant findings	no significant findings
c100	0.5	F	-	no significant findings	pneumonia - measles
c101	4.0	M	-	possible septicaemia	septicaemia
c104	6.0	M	-	cerebral malaria	malaria
c109	0.5	F	-	purulent meningitis	pneumonia

c117	2.9	M	-	cerebral malaria	malaria
c124	5.0	M	-	no significant findings	pneumonia
HIV negative cases for **Group B2** - c23,c50,c52,c57 and c93					
c5	1.7	F	+	no significant findings	CMV enteritis and pneumonia
c8	3.5	F	+	subtle widespread wm damage	pyothorax post measles
c9	5.1	F	+	medullitis	disseminated herpes
c16	5.3	F	+	no significant findings	pneumonia - interstitial
c24	5.0	M	+	measles encephalitis	pneumonia CMV disseminated
c51	1.8	F	+	low grade lymphocytic infiltrate	malaria fatty liver
c55	1.7	M	+	low grade lymphocytic infiltrate	pneumonia strongyloidosis
c62	4.0	F	+	low grade lymphocytic infiltrate	pneumonia
c70	2.1	F	+	low grade lymphocytic infiltrate	pneumonia
c82	0.2	F	+	cerebellar infarct & microhaemorrhages	pneumonia
c91	1.6	M	+	low grade lymphocytic infiltrate	pneumonia strongyloidosis
c110	0.4	M	+	toxoplasmosis	pneumonia - measles
c122	3.3	F	+	low grade lymphocytic infiltrate	pneumonia
c125	1.5	M	+	no significant findings	pneumonia
c126	1.5	F	+	no significant findings	TB - miliary
c129	0.4	F	+	low grade lymphocytic infiltrate	pneumonia
c131	0.4	M	+	low grade encephalitis	CMV enteritis
c140	4.0	M	+	no significant findings	pneumonia
c150	0.4	F	+	no significant findings	pneumonia
c157	0.2	M	+	purulent meningitis	pneumocystis pneumonia CMV
HIV positive cases for **Group B2** - c5,c125,c126, c140 and c150					

Sections were stained with Luxol fast blue (LFB) and assessed for overall staining on the light box (see Chapter 2), at a distance and in random distribution; on this basis they were graded into 3 categories:

Grade 1 – **marked** local or general pallor

Grade 2 – **moderate** local or general pallor

Grade 3 – **normal** intensity and distribution of LFB

The results for the 3 groups were then tabulated and compared with their ages.

Assessment of Immunohistochemistry

Sections were examined blinded as to age, sex, HIV status and recorded pathology findings. The immunostained sections were screened under low power to assess the general pattern of staining and then under high power to examine any specific areas of interest. Quantitative and/or qualitative assessment was then performed for the individual immunostains according to different protocols, depending on the complexity of the staining patterns.

The microscope used for routine screening was an Olympus BH2.

The image analysis equipment used was an Olympus BX40 microscope with an automatic stage video linked to a computer, using Image-Pro 4.5 software supplied by MediaCybernetics.

CD8: these sections were quantified by counting CD8 positive cells in the perivascular and parenchymal zones, in basal ganglia and hippocampus. As it was sometimes difficult to be certain of the exact location of the perivascular cells – some might have been intra-luminal and others intra-mural – the term "vessel associated cells" was used in some figures and tables. The counts were tabulated.

CD20: in these sections the CD20 positive cells were counted and tabulated for the perivascular zones and parenchymal zones of the basal ganglia and hippocampus.

CD14: these sections were assessed by a simple grading system for the whole section and tabulated;

1 = **sparse perivascular** cells

2 = **increased perivascular** cells but no parenchymal staining

3 = **slight parenchymal** staining

4 = **moderate parenchymal** staining

5 = **extensive and dense staining** with or without added local features, for example, nodules or necrosis.

CD16: these sections were assessed by image analysis and tabulated.

CD68: these sections were assessed by image analysis and tabulated.

HLA: the pattern of staining was found to be very similar to that for CD68 and no formal quantitation was performed.

MBP: these sections were screened under low power for distribution of staining.

βAPP: these sections were screened for the presence and distribution of staining and the results tabulated. A simple scoring system was adopted, from grades 1-3.

1 = **minimal** expression

2 = a **small** amount of positivity

3 = a **moderate** amount of staining either in extent or intensity.

GFAP: these sections were screened for evidence of increased GFAP activity in terms of intensity and distribution. The grading categories ranged from 1-4 and the results were tabulated.

1 = absent to **minimal** expression

2 = **slight** expression

3 = **moderate** expression

4 = **extensive** and strong expression

Image Analysis Protocol

A total sampling section area of 4sq. mm was chosen following discussion with experienced workers in this field. This equated well with the total area for 10 frames, using the x10 objective lens:

The single frame dimensions were -

Frame width = 740.441μm, height = 553.183μm and area = 409,585.3 sq.μm. Thus the total area for 10 frames = 4.095 sq. mm.

Generally, 5 separate areas were sampled with 2 frames, side-by-side, per area. Occasionally, if local tissue configuration dictated, for example if the internal capsule fascicles were especially narrow, a different frame arrangement was chosen. Sampling was as random and as wide as possible. The grey matter sampled was in the caudate nucleus and the white matter in the internal capsule. In the hippocampus, the grey matter sampled was that of the hippocampal and parahippocampal gyrus and the white matter from adjoining tracts.

An image was captured within the chosen area and the following steps were taken. The "count/size option" was selected from "measure" in the main soft-ware menu. In this dialogue box the "manual" option was chosen for the command "select colours". This opened another dialogue box named "segmentation". In this box, the sensitivity was set at 4. The stained objects or areas of interest were highlighted using an on-screen pointer and the sequential selection allowed the stained areas or objects to be

"filled" or captured. As regards cut-off, this was catered for by a reverse option that allowed a return to the previously selected stained areas if minimally stained or unwanted penumbral parts had been included. In this way staining that was considered as background could be largely excluded .

The dialogue box was then closed and on return to the count/size box the stained areas or objects were enumerated. Selection of the "view" option opened the "statistics" dialogue box where the individual areas and sum total were recorded. For each case, the result was expressed as the area stained in sq.μm per 4 sq.mm of section area scanned – which did not change between cases.

In counting cells, a total sampling area of 4sq. mm was chosen and determined by the use of an eyepiece graticule. Using a x20 objective lens, and a squared eyepiece graticule, a sample field had an area of 0.785 sq.mm. Five fields of 0.785sq.mm and one part field of 0.0754 sq.mm. gave a total area of 3.925 + 0.0754 = 4.0004 sq.mm. Random fields were selected by using out-of-focus movement in between counting areas.

4. 3 - Results

Overview of cases selected for this study

In tables 4.2.2 (basal ganglia) and 4.2.3 (hippocampus) the general neuropathology pathology findings are documented for the separate HIV negative and positive cases, showing that there are marked differences, as expected, between the two groups.

The group of **HIV negative** basal ganglia set included 4 cases of meningitis, 2 cases of possible septicaemia, 2 of malaria, and 11 cases for which there were no

significant findings. The group of **HIV positive** basal ganglia set included 2 cases of AIDS with toxoplasmosis, one of which showed HIVE at the original screening in 1997 (Bell et al. 1997), 1 case of meningitis, 1 case of measles encephalitis, 7 cases showing low grade lymphocytic infiltrate and 5 cases for which there were no significant findings.

For the hippocampus set, because some of the blocks corresponding to those of the basal ganglia set were unavailable, some substitutions were necessary. For example, the HIVE case **c152**, and a case showing low grade lymphocytic infiltrate, **c128**, from the basal ganglia set were replaced in the hippocampus set with two cases showing low grade lymphocytic infiltrate, **c51** and **c70**. Four of the cases showing a low grade lymphocytic infiltrate occurred in the youngest age group, aged 2 years or less. The sexes are almost equally distributed in the set, 23 females and 19 males.

Staining Results

Haematoxylin and Eosin: Review of these sections confirmed the presence of neuropathological findings reported in the original study (Figs 4.3.1 and 4.3.2). In the original study describing the neuropathology of this paediatric African cohort (Bell et al. 1997), white matter pallor or gliosis was noted in both HIV negative and positive cases.

Table 4.3.1 Number of cases showing white matter pallor/gliosis in original description (Bell et al. 1997)

HIV Negative Children		HIV Positive Children	
Age in months	Gliosis and/or Pallor	Age in months	Gliosis and/or Pallor
1-14m (n = 36)	12 **(33%)**	1-14m (n = 29)	6 **(21%)**
>15m (n = 42)	14 **(33%)**	> 15m (n = 48)	7 **(15%)**

Luxol Fast Blue: These stained sections were very useful for displaying the general tissue architecture. There was naturally a variation in tissue block boundaries given the age range and the difficulties under which the post mortems were performed. In the basal ganglia set, the internal capsule was present in all of the sections but other structures, such as the corpus callosum, external capsule, septum and anterior commissure, were variable in their inclusion. The comparison of sections on the light box was used for an overall assessment of pallor. The results are shown in **Appendices 11 and 12**.

Table 4.3.2 - Results Mann-Whitney tests for LFB grades

LFB Grades					
Basal Ganglia			**Hippocampus**		
Group A1			Group A2		
(n=40)	medians	p values	(n=40)	medians	p values
HIV –ve	2		HIV -ve	2	
HIV +ve	2	p=0.666	HIV +ve	2	p=0.841
Group B1 (n=5)			Group B2 (n=5)		
HIV –ve	3		HIV –ve	2	
HIV +ve	2	p=0.212	HIV +ve	2	p=1.000

Table 4.3.2 shows the summary statistics for the LFB grades and no significant difference was detected between HIV negative and positive grades in any of the groups.

Myelin Basic Protein: The sections stained with myelin basic protein displayed the white matter in the same way as Luxol fast blue and screening of these sections provided no additional information.

β-APP: The grades for βAPP positivity are shown in **Appendices 13 and 14**, and Fig 4.3.3 shows the pattern of staining when present.. Only about a third of HIV negative and HIV positive cases showed evidence of βAPP reactivity and, in these, it was mostly of limited extent and positivity – no tabulation is provided here but it should be noted (see Appendix 13) that for the six HIV negative and six HIV positive caes showing staining in the basal ganglia the grading results were significantly

greater for the HIV negative cases p=0.029 (Mann-Whitney). In general, HIV negative cases showed more positivity than HIV positive cases. There was no obvious correlation with pathology, except that the case with meningoencephalitis and infarcts, **c54**, showed grade 3 βAPP positivity. There was no obvious relationship with GFAP reactivity. Only one of the two malaria cases was positive. Case **c93** showed neuronal positivity in the cortex and in the caudate nucleus.

Glial Fibrillary Acidic Protein: The general staining was satisfactory, all but one section showing positivity. The pattern varied from a delicate pattern of restricted expression, as in **c42**, to a strong and generalised reaction, Grade 4, as in **c121**, in which there was pronounced staining in the grey matter as well. The results of grading were tabulated (**Appendices 15 and 16**). The pattern of GFAP positivity is shown in Figs 4.3.4 and 4.3.5. The results of Mann-Whitney testing for GFAP positivity in the basal ganglia (Group A1) and hippocampus (Group A2) of the 20 HIV negative and 20 HIV positive cases, and the subsets lacking pathology (Groups B1 and B2) are shown in Table 4.3.2.and no significant differences were detected. The means for the HIV positive cases were slightly greater than the corresponding means for HIV negative cases in the basal ganglia, HIV positive 1.9 (0.79), HIV negative 1.55 (0.69), and in the hippocampus, HIV positive 2.65 (1.09), HIV negative 2 (0.95), which is suggestive of a slight trend but this did not reach significance.

Table 4.3.3 - Results of Mann-Whitney tests for GFAP grades

GFAP Grades					
Basal Ganglia			**Hippocampus**		
Group A1			Group A2		
(n=40)	medians	p values	(n=40)	medians	p values
HIV –ve	1		HIV -ve	2	
HIV +ve	2	p=0.151	HIV +ve	2.5	p=0.120
Group B1 (n=5)			Group B2 (n=5)		
HIV –ve	2		HIV –ve	1	
HIV +ve	1	p=0.908	HIV +ve	4	p=0.154

Table 4.3.3 shows the summary statistics for the GFAP grading and no significant difference between HIV negative and positive cases was detected in any of the groups.

Table 4.3.4 Basal ganglia - combined CD results

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	**BG - combined CD results**																		
2	**Code**	**Age**		**HIV**	**CD8**				**CD20**				**CD14**	**CD16**		**CD68**		**CNS Pathology findings**	**Causes of death**
3	**HIV negative cases**				**gm-pvc**	**gm-par**	**wm-pvc**	**wm-par**	**gm-pvc**	**gm-par**	**wm-pvc**	**wm-par**	**grd**	**gm**	**wm**	**gm**	**wm**	**on routine staining**	
4	c33	1.6	F	-	4	0	6	0	2	0	0	0	3	12	51	1,211	1,088	possible septicaemia	pulmonary infarction
5	c42	5.0	F	-	*	*	*	*	2	0	0	0	2	1	33	923	363	no significant findings	pneumonia
6	c50	1.6	F	-	16	0	18	3	4	0	9	0	2	*	*	364	464	no significant findings	pneumonia malaria
7	c54	0.4	M	-	5	0	5	0	5	0	10	0	5	37	82	310	763	meningoencephalitis and infarcts	no significant findings
8	c57	5.0	F	-	8	0	8	0	9	1	4	0	1	18	1	313	233	no significant findings	lymphoma - B-cell
9	c59	1.6	M	-	4	3	4	0	2	0	2	0	1	*	*	168	64	purulent meningitis	malnutrition
10	c60	1.5	F	-	10	0	6	0	17	1	20	0	2	18	34	634	440	no significant findings	malaria pulmonary infarction
11	c67	4.0	F	-	17	1	13	5	0	0	0	0	2	61	26	640	989	no significant findings	pneumonia
12	c74	1.5	F	-	1	0	0	0	0	0	3	0	2	68	99	988	523	no significant findings	pneumonia
13	c76	1.6	M	-	5	0	6	0	2	0	0	0	1	12	39	548	383	no significant findings	colitis
14	c77	0.3	F	-	16	0	16	0	1	0	0	0	4	14	68	1,887	1,231	possible septicaemia	sickle cell crisis
15	c89	0.5	F	-	10	2	12	2	0	0	0	0	3	17	24	390	494	purulent meningoencephalitis	pulmonary abscess
16	c93	1.5	F	-	3	0	2	0	0	0	0	0	2	0	16	261	126	no significant findings	colitis
17	c94	0.5	M	-	4	0	5	0	0	0	0	0	2	1	1	250	225	no significant findings	no significant findings
18	c100	0.5	F	-	*	*	*	*	0	0	0	0	1	*	*	225	131	no significant findings	pneumonia - measles
19	c101	4.0	M	-	4	1	2	0	0	0	0	0	4	35	1	549	505	possible septicaemia	septicaemia
20	c104	6.0	M	-	17	0	5	1	0	0	0	0	4	27	1	676	341	cerebral malaria	malaria
21	c109	0.5	F	-	0	0	2	0	1	0	2	0	3	37	32	118	105	purulent meningitis	pneumonia
22	c113	0.4	M	-	3	0	7	0	5	0	1	0	1	54	88	14	211	no significant findings	pneumonia
23	c117	2.9	M	-	11	0	2	0	2	0	3	0	3	10	22	370	185	cerebral malaria	malaria
24																			
25	Means				7.6	0.4	6.6	0.6	2.6	0.1	2.7	0	2.4	25	36	542	443		
26	SD				5.7	0.8	5.1	1.4	4.1	0.3	5	0	1.2	21	31	441	336		
27																			
28	**HIV positive cases**																		
29	c5	1.7	F	+	15	3	20	1	7	0	1	0	3	*	*	1,581	1,952	no significant findings	CMV enteritis and pneumonia
30	c8	3.5	F	+	18	0	25	17	1	0	0	0	2	14	598	1,394	1,030	subtle widespread wm damage	pyothorax post measles
31	c9	5.1	F	+	10	0	6	2	2	0	0	0	1	50	116	1,027	1,100	medullitis	disseminated herpes zoster
32	c24	5.0	M	+	19	1	10	4	0	0	0	0	3	41	300	2,180	1,435	measles encephalitis	pneumonia CMV disseminated
33	c55	1.7	M	+	38	0	32	8	8	0	7	0	1	637	25	1,098	712	low grade lymphocytic infiltrate	pneumonia strongyloidosis
34	c62	4.0	F	+	25	0	38	81	3	0	10	0	1	1	14	698	592	low grade lymphocytic infiltrate	pneumonia
35	c82	0.2	F	+	1	0	0	0	0	0	0	0	3	0	12	1,050	811	cerebellar infarct & microhaemorrhages	pneumonia
36	c91	1.6	M	+	47	70	64	206	0	0	0	0	2	*	*	1,592	1,193	low grade lymphocytic infiltrate	pneumonia strongyloidosis
37	c110	0.4	M	+	49	135	50	213	0	0	0	0	4	31	395	1,125	985	toxoplasmosis	pneumonia - measles
38	c121	1.6	M	+	7	0	4	0	0	0	0	0	3	1	16	431	420	cerebral oedema	pneumonia
39	c122	3.3	F	+	30	0	43	1	4	0	2	0	4	10	812	1,672	1,628	low grade lymphocytic infiltrate	pneumonia
40	c125	1.5	M	+	25	0	17	13	3	0	1	0	2	10	491	1,490	698	no significant findings	pneumonia
41	c126	1.5	F	+	17	1	23	10	1	0	1	0	1	16	32	298	269	no significant findings	TB - miliary
42	c128	1.8	F	+	46	1	62	93	4	0	2	0	4	100	1343	725	187	low grade lymphocytic infiltrate	pneumonia
43	c129	0.4	F	+	49	3	30	54	3	0	6	0	3	31	46	550	433	low grade lymphocytic infiltrate	pneumonia
44	c131	0.4	M	+	5	8	16	13	8	0	0	0	3	70	531	206	407	low grade encephalitis	CMV enteritis
45	c140	4.0	M	+	39	0	12	10	0	0	0	0	2	15	706	412	364	no significant findings	pneumonia
46	c150	0.4	F	+	10	5	23	20	3	0	0	0	3	68	95	1,076	812	no significant findings	pneumonia
47	c152	6.4	M	+	18	53	80	312	1	0	33	0	5	25	3555	741	348	HIV encephalitis & toxoplasmosis	pneumonia, interstitial
48	c157	0.2	M	+	20	40	20	53	7	0	6	0	4	1014	1500	1,408	922	purulent meningitis	pneumocystis pneumonia CMV
49																			
50	Means				24	16	29	56	2.8	0	3.5	0	2.7	119	588	1,038	815		
51	SD				15	34	22	88	2.8	0	7.5	0	1.2	267	867	530	476		
52																			

Table 4.3.5 Hippocampus - combined CD results

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	**Hippocampus - combined CD results**																		
2	**Code**	**Age**	**Sex**	**HIV**	**CD8**				**CD20**				**CD14**	**CD16**		**CD68**		**CNS pathology findings**	**Causes of death**
3					**gm-pvc**	**gm-par**	**wm-pvc**	**wm-par**	**gm-pvc**	**gm-par**	**wm-pv**	**wm-par**	**grd**	**gm**	**wm**	**gm**	**wm**	**on routine staining**	
4	c23	1.5	M	-	3	0	22	2	5	0	1	1	1	11	948	807	568	no significant findings	enteritis pulmonary CMV
5	c33	1.6	F	-	2	1	6	0	6	0	2	0	1	106	38	3147	2169	possible septicaemia	pulmonary infarction
6	c50	1.6	F	-	5	0	14	3	2	0	14	0	2.5	17	16	155	244	no significant findings	pneumonia malaria
7	c52	0.5	M	-	1	0	2	0	0	0	0	0	2	28	32	49	209	no significant findings	pneumonia
8	c54	0.4	M	-	2	0	17	0	0	0	30	0	5	341	239	966	1103	meningoencephalitis & infarcts	no significant findings
9	c57	5.0	F	-	8	1	22	0	4	1	3	1	1	11	10	157	394	no significant findings	lymphoma - B-cell
10	c59	1.6	M	-	5	0	14	0	0	0	5	0	1	27	0	780	491	purulent meningitis	malnutrition
11	c60	1.5	F	-	0	0	0	0	11	0	18	0	2.5	27	36	182	1295	no significant findings	malaria pulmonary infarction
12	c63	2.5	F	-	2	0	9	0	6	0	4	0	1	15	15	1111	249	no significant findings	pneumonia colitis
13	c77	0.3	F	-	10	0	9	0	2	0	1	0	4.5	97	255	1162	1362	possible septicaemia	sickle cell crisis
14	c86	1.8	F	-	3	0	5	0	5	0	15	0	1	145	32	149	139	no significant findings	pneumonia
15	c89	0.5	F	-	4	0	15	2	1	1	6	0	2	31	12	116	407	purulent meningoencephalitis	pulmonary abscess
16	c93	1.5	F	-	1	0	3	0	0	0	0	0	2.5	214	18	614	105	no significant findings	colitis
17	c94	0.5	M	-	2	0	14	0	0	0	1	0	2	74	66	575	122	no significant findings	no significant findings
18	c100	0.5	F	-	5	1	7	1	0	0	2	0	2	19	43	436	426	no significant findings	pneumonia - measles
19	c101	4.0	M	-	20	21	9	3	0	0	1	0	3	69	203	839	1406	possible septicaemia	septicaemia
20	c104	6.0	M	-	12	4	18	1	5	0	3	0	3.5	71	52	2356	1256	cerebral malaria	malaria
21	c109	0.5	F	-	0	0	2	0	0	0	0	0	3	31	19	163	349	purulent meningitis	pneumonia
22	c117	2.9	M	-	3	1	4	0	2	0	6	0	3	166	114	984	870	cerebral malaria	malaria
23	c124	5.0	M	-	7	0	9	4	1	0	0	0	2.5	41	75	1162	549	no significant findings	pneumonia
24																			
25	Means				5	1	10	1	2.5	0.1	5.6	0.1	2.3	77	111	796	686		
26	SD				5	5	7	1	3	0.3	7.8	0.3	1.2	84	211	783	565		
27																			
28	c5	1.7	F	+	10	0	22	4	0	0	5	0	3.5	161	745	1751	816	no significant findings	CMV enteritis and pneumonia
29	c8	3.5	F	+	1	1	5	1	2	0	3	0	2	1053	385	1698	1529	subtle widespread wm damage	pyothorax post measles
30	c9	5.1	F	+	7	2	21	4	2	0	0	0	1	123	178	476	858	medullitis	disseminated herpes zoster
31	c16	5.3	F	+	8	3	26	1	0	0	0	0	1	257	101	662	810	no significant findings	pneumonia - interstitial
32	c24	5.0	M	+	8	0	32	2	0	0	1	0	3	118	215	4995	1003	measles encephalitis	pneumonia CMV disseminated
33	c51	1.8	F	+	12	2	48	4	7	1	6	0	2	12	29	399	331	low grade lymphocytic infiltrate	malaria fatty liver
34	c55	1.7	M	+	14	2	41	1	1	0	8	1	3	28	133	534	427	low grade lymphocytic infiltrate	pneumonia strongyloidosis
35	c62	4.0	F	+	24	13	51	23	4	0	18	1	1	13	55	621	361	low grade lymphocytic infiltrate	pneumonia
36	c70	2.1	F	+	60	25	87	48	7	1	24	2	2	22	43	727	1564	low grade lymphocytic infiltrate	pneumonia
37	c82	0.2	F	+	0	0	0	0	0	0	1	0	4.5	408	402	1014	736	cerebellar infarct & microhaemorrhages	pneumonia
38	c91	1.6	M	+	114	41	244	85	10	0	16	0	2	382	4792	4047	1500	low grade lymphocytic infiltrate	pneumonia strongyloidosis
39	c110	0.4	M	+	117	114	196	98	1	0	2	0	3.5	186	236	1134	1477	toxoplasmosis	pneumonia - measles
40	c122	3.3	F	+	28	6	40	0	0	0	3	0	4	1120	1239	1932	2310	low grade lymphocytic infiltrate	pneumonia
41	c125	1.5	M	+	5	0	24	5	1	0	1	0	2	120	209	1236	885	no significant findings	pneumonia
42	c126	1.5	F	+	2	0	14	0	0	0	1	0	2	29	311	454	477	no significant findings	TB - miliary
43	c129	0.4	F	+	17	10	48	42	5	0	4	1	3	405	207	925	1229	low grade lymphocytic infiltrate	pneumonia
44	c131	0.4	M	+	16	0	20	0	0	0	1	0	2	182	33	474	247	low grade encephalitis	CMV enteritis
45	c140	4.0	M	+	9	4	32	10	0	0	0	0	2	12	44	593	810	no significant findings	pneumonia
46	c150	0.4	F	+	6	2	33	8	0	0	2	0	3	49	16	716	661	no significant findings	pneumonia
47	c157	0.2	M	+	15	21	20	10	2	1	9	1	5	663	712	2715	1715	purulent meningitis	pneumocystis pneumonia CMV
48																			
49	Means				24	12	50	17	2.1	0.15	5.25	0.3	2.5	267	504	1355	987		
50	SD				34	26	62	29	3	0.4	7	0.6	1	329	1055	1250	548		
51																			

Notes for Tables 4.3.4 and 4.3.5

1. Key – bg=basal ganglia, hippo=hippocampus, grd=grade, gm=grey matter, wm=white matter, pvc=perivascular cells and par=parenchymal cells
2. Asterisks,*, denote values not available for technical reasons
3. Decimal points in means and standard deviations generally to first point if value below 10 otherwise to nearest whole number
4. For some CD14 cases it was difficult to assign a grade because of marked variation within the section – in these cases an intermediate grade was given, e.g. 4.5.and the default value would be the upper grade e.g.
5. Standard deviations are given in brackets after the means e.g.7.6 (5.7)

CD8: The results of cell counts are shown in Tables 4.3.4 and 4.3.5 and staining patterns are illustrated in Figs 4.3.6 - 4.3.8. **Perivascular CD8 cells** were identified and counted in almost all **HIV negative cases**, in basal ganglia and hippocampus, and for both grey matter and white matter. The mean counts, with standard deviations, for the basal ganglia grey and white matter were approximately equal, respectively 7.6 (5.7) and 6.6 (5.1), but in the hippocampus the counts for the white matter were appreciably higher - 10 (7) compared with 5 (5). An elevated count in the grey matter was not necessarily accompanied by a similar rise in the white matter, and vice versa, but quite often the counts are approximately equal. In the basal ganglia set, 7 cases showed counts of 10 or greater. In the hippocampus, 10 cases show counts of 10 or more for the grey matter or white matter, or both. Some

of these are cases in common with the basal ganglia set but there are differences, for example in respect of the possible septicaemia cases – one showing higher values in the basal ganglia set and the other in the hippocampus set. **Parenchymal CD8 cells** were found in very few basal ganglia - in the white matter in five cases and in the grey, in 4 cases. Most of these cases also showed higher perivascular counts. In the hippocampus, the situation was much the same except there was a high count in the grey matter case, **c101**, which had signs of possible septicaemia. **Perivascular cells** in the basal ganglia of **HIV positive cases** showed approximately equal counts in grey and white matter. The respective means and standard deviations were 24 (15) and 29 (22). Cases previously recorded as having a low grade lymphocytic infiltrate, and the AIDS case with toxoplasmosis, **c110**, showed particularly high CD8 counts in the grey matter but so also did 2 cases with no recorded pathology. Generally the counts for the white matter mirrored the grey matter findings. Counts for the HIVE case, **c152**, proved to be the highest for both grey and white matter. The lowest counts were seen in **c152**, which had a cerebellar infarct and microhaemorrhages, and in **c121**, which displayed cerebral oedema. A case with subtle widespread white matter damage, **c8**, which proved later to have very high values in the CD68 results, showed some elevation in the CD8 counts, but only in the basal ganglia. The counts for the measles encephalitis case, **c24**, were below the means for grey matter and white matter, for both the basal ganglia and hippocampus. In the hippocampus, the mean counts for the grey matter, 24 (34) and white matter, 50 (62) differed appreciably. The higher counts in the grey matter, like those in the basal ganglia, were seen in the low grade lymphocytic infiltrate cases and, much higher, in the AIDS case, **c110.** The higher perivascular counts in the white matter generally reflect

those in the grey but in some cases were appreciably greater, as in **c91**, a count of 244 against 114, and for **c110**, a count of 196 against 117. The standard deviations are greater than the means for the CD8 counts in the hippocampus. **Parenchymal CD8 counts** in the grey and white matter of the basal ganglia were high but were even more numerous in the white matter, 16 (34) and 56 (88) respectively. The corresponding means for the hippocampus are 12 (26) and 17 (29). In all of these counts, the standard deviations are greater than the means. Four cases showed particularly high parenchymal counts in the grey matter. Two of these were AIDS cases (**c110** and **c152**), **c91** had a low-grade lymphocytic infiltrate, and **c157** had purulent meningitis. Each of these had even higher counts in the white matter, some by a factor of 2 or more. Three other cases had counts greater than 50 and all had low-grade lymphocytic infiltrate cases. In the hippocampus, the highest count for the grey matter was 114, in the AIDS case, **c110**. The other cases with counts above the mean of 12 (26) included 3 cases of low-grade lymphocytic infiltrate and the case of purulent meningitis, **c157**. White matter parenchymal counts revealed 4 cases that had values above the mean of 17 (29), one being the AIDS case, **c110,** as in the grey matter counts above, and the other 3 all showed a low-grade lymphocytic infiltrate.

The Mann-Whitney tests (Table 4.3.6) comparing the counts for **perivascular CD8 cells** in HIV negative and positive groups**,** confirmed that they were significantly greater in HIV positive basal ganglia in Group A1, (gm $p=0.001$ & wm $p=0.000$) and for **parenchymal CD8 cells,** in both Group A1 (gm $p=0.001$ & wm $p= 0.000$) and in Group B1 for white matter only ($p=0.034$).

Results for the hippocampus (Groups A2 and B2) were similar. In Group A2, **perivascular CD8 cells** were significantly more numerous in both grey and white

matter (gm p=0.002, wm=0.006) as were **parenchymal CD8 cells** (gm p=0.003, wm p=0.006). In Group B2, a significant increase in HIV positive cases was detected for **parenchymal CD8 cells** only in the white matter (p=0.011). In Table 4.3.6, perivascular is abbreviated to pv, and parenchymal to par, and significant values are shown in bold type.

Figs 4.3.19, 4.3 20, 4.3 29 and 4.3.30 show these results in the form of boxplots for basal ganglia and hippocampus. The scatterplots, Figs 4.3.39 and 4.3.40 show reasonable correlation between the grey and white matter for both basal ganglia and hippocampus but are distorted by outlying values.

Table 4.3.6 – Mann-Whitney test results for CD8 counts: basal ganglia and hippocampus

CD8 Counts				**CD8 Counts**			
Basal ganglia Mann-Whitney tests				**Hippocampus Mann-Whitney tests**			
		gm medians	wm medians			gm medians	wm medians
Group A1	HIV -ve	5	5	Group A2	HIV -ve	3	9
pv cells	HIV +ve	19.5	23	pv cells	HIV +ve	11	32
	p value	**p=0.001**	**p=0.000**		p value	**p=0.002**	**p=0.000**
		gm medians	wm medians			gm medians	wm medians
Group A1	HIV -ve	0	0	Group A2	HIV -ve	0	0

par cells	HIV +ve	1	13	par cells	HIV +ve	2	4
	p value	**p=0.011**	**p=0.000**		p value	**p=0.003**	**p=0.006**
		gm medians	wm medians			gm medians	wm medians
Group B1	HIV -ve	5	7	Group B2	HIV -ve	3	14
pv cells	HIV +ve	17	20	pv cells	HIV +ve	6	24
	p value	**p=0.115**	**p=0.060**		p value	**p=0.172**	**p=0.071**
		gm medians	wm medians			gm medians	wm medians
Group B1	HIV -ve	0	0	Group B2	HIV -ve	0	0
par cells	HIV +ve	1	10	par cells	HIV +ve	0	21
	p value	**p=0.251**	**p=0.034**		p value	**p=0.441**	**p=0.011**

CD20: Results are shown in Tables 4.3.4 and 4.3.5 and are summarised in Table 4.3.7. CD positive B lymphocytes (Fig.4.3.9) were counted in a total area of 4 sq. mm. and in two categories, perivascular cells and parenchymal cells. The perivascular group should more correctly be termed "vessel associated cells" in view of the difficulty in determining the exact relationship of any particular cell with the vessel wall of capillaries. In the basal ganglia of **HIV negative cases** there was a very limited presence of B-cells, both in grey and white matter. **Perivascular CD20 cells** were detected more frequently in the white matter than in grey, with means (standard deviations) of 5.6 (7.8) and 2.5 (3) respectively. In this group the only high value was detected in a case of meningoencephalitis, **c54**. A similar response was seen in the hippocampus set, where the white matter mean was 5.25 (7) against that

for the grey matter 2.1 (3). The only other case with high values and common to both was **c60**, a case reported previously as having no significant findings. A few positive cells were detected in one of the cases of malaria in the basal ganglia set, **c117**, and in both in the hippocampus set. In general, **parenchymal CD20 cells** were rarely seen and no formal analysis was attempted. In the basal ganglia of the **HIV positive cases**, the highest count in the white matter was in the HIVE case, and lower counts were seen in the low-grade lymphocytic infiltrate cases and case **c157** , with purulent meningitis. No CD20 cells were seen in the other AIDS case, **c110**, or in the measles encephalitis case. In the hippocampus, higher counts are seen in the cases showing low-grade lymphocytic infiltrate. The counts for the AIDS case, **c110,** and for the measles encephalitis case were minimal.

As regards analysis, it should be noted that the means for the grey matter vary little between HIV positive and negative cases, in both the basal ganglia and hippocampus, all being in the 2-3 range. For the white matter, mean values in the basal ganglia are slightly less than those for the hippocampus, where the mean for HIV negative cases is slightly more that for the HIV positive cases, 5.6 (7.8) as opposed to 5.25 (7). The results are shown as boxplots in Figs 4.3.21, 4.3.22, 4.3.31 and 4.3.32. No result of statistical significance was recorded in the CD20 tables for either basal ganglia or hippocampus – see Table 4.3.7.

Table 4.3.7 - Mann-Whitney test results for CD20 counts: basal ganglia and hippocampus

CD20 Counts				CD20 Counts			
Basal ganglia Mann-Whitney tests				Hippocampus Mann-Whitney tests			
		gm medians	wm medians			gm medians	wm medians
Group A1	HIV -ve	1.5	0	Group A2	HIV -ve	1.5	2.5
pv cells	HIV+ve	2.5	0.5	pv cells	HIV+ve	1	2.5
		p=0.471	**p=0.895**			**p=0.651**	**p=0.913**
		gm medians	wm medians			gm medians	wm medians
Group B1	HIV -ve	2	0	Group B2	HIV -ve	2	1
pv cells	HIV +ve	3	1	pv cells	HIV +ve	0	1
		p=0.832	**p=0.908**			**p=0.158**	**p=0.295**

CD14: The results are shown in Tables 4.3.4 and 4.3.5 and summarised in Table 4.3.8. For CD14 the staining was generally satisfactory (Fig. 4.3.10). In the **HIV negative cases**, the CD14 grades for the basal ganglia and hippocampus are quite similar for those cases that were common to Groups A1 and A2, and the means (standard deviations) were 2.4 (1.2) and 2.3 (1.2) respectively. Generally the higher grades of staining were related to the presence of significant pathology. In particular, c**54**, a case with meningoencephalitis and infarcts, had a score of 5 for both basal ganglia and hippocampus. The meningitis and malaria cases had scores above the means in both sets, as did the possible septicaemia cases. In the basal ganglia of the **HIV positive cases**, the mean score was 2.7 (1.2) and for the hippocampus, 2.5 (1). The highest grades in both regions were in the AIDS cases, **c82** with cerebellar

infarcts and microhaemorrhages and **c157** with purulent meningitis. The grades for the measles encephalitis case were likewise above the means. The cases with low grade lymphocytic infiltration had scores in the middle to lower range.

Table 4.3.8 - CD14 grades: basal ganglia and hippocampus

CD14 Grades			CD14 Grades		
Basal Ganglia Mann-Whitney test			Hippocampus Mann-Whitney test		
	HIV status	medians		HIV status	means
Group A1	HIV –ve HIV +ve	2.00 3.00 p=0.388	Group A2	HIV –ve HIV +ve	2.25 2.00 p=0.49
Group B1	HIV –ve HIV +ve	1.6 2.2 p=0.374	Group B2	HIV –ve HIV +ve	2 2.6 p=0.208

The CD 14 results are also displayed in the boxplots, Figs 3.3.23, 3.3.24, 3.3.33 and 3.3.34.

CD16: The results are shown in Tables 4.3.4 and 4.3.5 and summarised in Table 4.3.9 and illustrated in. Staining quality was generally satisfactory (Fig 4.3.11) but in 5 cases, 3 HIV negative and 2 positive, the staining in the basal ganglia was so poor as to preclude analysis. To balance the sets for analysis, one HIV positive case, **c82**,

aged 0.2 years and with an original diagnosis of cerebellar infarct and microhaemorrhages, was removed. This gave 17 pairs for comparison for CD16 staining in the basal ganglia, a subset of Group A1 designated Group C (n=34). For CD16 staining comparisons in the hippocampus, Group A2 was used, as for all other antibody screening.

The results for CD16 staining in the basal ganglia of the **HIV negative cases** showed mean values (standard deviations) in the white matter of 36 (31), and in the grey matter, 25 (21). For the hippocampus, the corresponding means and standard deviations were, for the white matter, 111 (211), and for the grey matter, 77 (84). In the basal ganglia, two of the cases with higher values, **c93** and **c113**, had shown no specific pathology on routine examination and another two, **c33** and **c77**, were possible septicaemia cases. Case **c54**, with meningoencephalitis & infarcts, also showed higher values. The values for the malaria cases showed little difference from the means. The reason for the difference in mean values between the basal ganglia and the hippocampus is uncertain. It is possible that suboptimal staining in the basal ganglia (which had made necessary the creation of a smaller subset, Group C) was a contributory factor. Among the cases that were common to Groups C and A2, those with high CD16 values in the hippocampus generally paralleled those with elevated values in the basal ganglia, except that the values were higher in Group A2. A notable difference was that in the hippocampus, the values for one of the malaria cases, **c104** with a grey matter value of 27 and a white matter value of 36**,** were little different from, or even below, the means, while the other, **c117**, showed a slight increase (grey matter 166 and white matter 114). In the **HIV positive cases**, in both basal ganglia and the hippocampus, the white matter generally shows a greater

degree of activation than the grey. In the basal ganglia, the mean value (standard deviation) for the white matter was 588 (867) and for the grey matter, 119 (267). In the hippocampus, the corresponding values for white matter were 504 (1055) and for grey, 267 (329).

Nevertheless, some very high values were seen for the white matter in the basal ganglia, especially for the HIVE case, **c152**, (3555), **c157**, with purulent meningitis (1500), and **c128**, one of the cases showing low grade lymphocytic infiltrate (1343) . Others in this category showed little evidence of activation apart from a high value for the grey matter of **c55** (637). The other findings of note were the relatively low values for the other AIDS case, **c110**, 31 and 395 for grey and white matter respectively, and the measles encephalitis case, **c24**, 41 for grey matter, and 300 for white matter.

In the hippocampus set, the higher values were generally in the cases that were common to both sets, for example cases **c8**, (1053 and 385), and **c157**, (633 and 712) for grey and white matter respectively. The same was true for the lower values for the case with measles encephalitis and for the AIDS case, **c110**, 186 and 236 respectively for grey and white matter.

The differences for CD16 staining values, between HIV positive and negative cases, are statistically significant for the white matter in the basal ganglia, $p=0.030$, and the grey matter and white matter in the hippocampus, $p=0.037$ and $p=0.004$ respectively. However, comparison of HIV positive and negative cases in the small subsets, Groups B1 and B2, failed to reveal significant differences for CD16 staining values.

The CD16 results are also displayed in the boxplots, Figs 4.3.25, 4.3.26, 4.3.35 and 4.3.36.

Table 4.3.9 – Mann-Whitney results for CD16 image analysis: basal ganglia and hippocampus

CD16 image analysis				**CD16 image analysis**			
Basal ganglia Mann-Whitney tests				**Hippocampus Mann-Whitney tests**			
		gm medians	wm medians			gm medians	wm medians
Group C	HIV -ve	18	32	Group A2	HIV -ve	36	37
	HIV +ve	28	347.5		HIV +ve	142	208
	p value	**p=0.447**	**p=0.03**		p value	**p=0.037**	**p=0.004**
		gm medians	wm medians			gm medians	wm medians
Group B1	HIV -ve	18	26	Group B2	HIV -ve	17	18
	HIV +ve	15	95		HIV +ve	49	209
	p value	**p=0.753**	**p=0.210**		p value	**p=0.295**	**p=0.346**

Table 4.3.10 - CD68 results for the basal ganglia

BG - combined CD results							
Code	**Age**		**HIV**	**CD14**	**CD68**		**CNS Pathology findings on routine Staining**
HIV negative cases				**grd**	**gm**	**wm**	
c33	1.6	F	-	3	1,211	1,088	possible septicaemia
c42	5.0	F	-	2	923	363	no significant findings
c50	1.6	F	-	2	364	464	no significant findings
c54	0.4	M	-	5	310	763	meningoencephalitis and infarcts
c57	5.0	F	-	1	313	233	no significant findings
c59	1.6	M	-	1	168	64	purulent meningitis
c60	1.5	F	-	2	634	440	no significant findings
c67	4.0	F	-	2	640	989	no significant findings
c74	1.5	F	-	2	988	523	no significant findings
c76	1.6	M	-	1	548	383	no significant findings
c77	0.3	F	-	4	1,887	1,231	possible septicaemia
c89	0.5	F	-	3	390	494	purulent meningoencephalitis
c93	1.5	F	-	2	261	126	no significant findings
c94	0.5	M	-	2	250	225	no significant findings
c100	0.5	F	-	1	225	131	no significant findings
c101	4.0	M	-	4	549	505	possible septicaemia
c104	6.0	M	-	4	676	341	cerebral malaria
c109	0.5	F	-	3	118	105	purulent meningitis
c113	0.4	M	-	1	14	211	no significant findings
c117	2.9	M	-	3	370	185	cerebral malaria
means				2.4	542	443	
SD				1.2	441	336	
HIV positive cases				**grd**	**gm**	**wm**	

c5	1.7	F	+	3	1,581	1,952	no significant findings
c8	3.5	F	+	2	1,394	1,030	subtle widespread wm damage
c9	5.1	F	+	1	1,027	1,100	medullitis
c24	5.0	M	+	3	2,180	1,435	measles encephalitis
c55	1.7	M	+	1	1,098	712	low grade lymphocytic infiltrate
c62	4.0	F	+	1	698	592	low grade lymphocytic infiltrate
c82	0.2	F	+	3	1,050	811	cerebellar infarct & microhaemorrhages
c91	1.6	M	+	2	1,592	1,193	low grade lymphocytic infiltrate
c110	0.4	M	+	4	1,125	985	toxoplasmosis
c121	1.6	M	+	3	431	420	cerebral oedema
c122	3.3	F	+	4	1,672	1,628	low grade lymphocytic infiltrate
c125	1.5	M	+	2	1,490	698	no significant findings
c126	1.5	F	+	1	298	269	no significant findings
c128	1.8	F	+	4	725	187	low grade lymphocytic infiltrate
c129	0.4	F	+	3	550	433	low grade lymphocytic infiltrate
c131	0.4	M	+	3	206	407	low grade encephalitis
c140	4.0	M	+	2	412	364	no significant findings
c150	0.4	F	+	3	1,076	812	no significant findings
c152	6.4	M	+	5	741	348	HIV encephalitis & toxoplasmosis
c157	0.2	M	+	4	1,408	922	purulent meningitis
means				2.7	1,038	815	
SD				1.2	530	476	

Table 4.3.11 CD68 results for the hippocampus

Hippocampus - combined CD results						
Code	**Age**	**Sex**	**HIV**	**CD68**		**CNS pathology findings**
				gm	**wm**	**on routine staining**
c23	1.5	M	-	807	568	no significant findings
c33	1.6	F	-	3147	2169	possible septicaemia
c50	1.6	F	-	155	244	no significant findings
c52	0.5	M	-	49	209	no significant findings
c54	0.4	M	-	966	1103	meningoencephalitis & infarcts
c57	5.0	F	-	157	394	no significant findings
c59	1.6	M	-	780	491	purulent meningitis
c60	1.5	F	-	182	1295	no significant findings
c63	2.5	F	-	1111	249	no significant findings
c77	0.3	F	-	1162	1362	possible septicaemia
c86	1.8	F	-	149	139	no significant findings
c89	0.5	F	-	116	407	purulent meningoencephalitis
c93	1.5	F	-	614	105	no significant findings
c94	0.5	M	-	575	122	no significant findings
c100	0.5	F	-	436	426	no significant findings
c101	4.0	M	-	839	1406	possible septicaemia
c104	6.0	M	-	2356	1256	cerebral malaria
c109	0.5	F	-	163	349	purulent meningitis
c117	2.9	M	-	984	870	cerebral malaria
c124	5.0	M	-	1162	549	no significant findings
means				796	686	
SD				783	565	

c5	1.7	F	+	1751	816	no significant findings
c8	3.5	F	+	1698	1529	subtle widespread wm damage
c9	5.1	F	+	476	858	medullitis
c16	5.3	F	+	662	810	no significant findings
c24	5.0	M	+	4995	1003	measles encephalitis
c51	1.8	F	+	399	331	low grade lymphocytic infiltrate
c55	1.7	M	+	534	427	low grade lymphocytic infiltrate
c62	4.0	F	+	621	361	low grade lymphocytic infiltrate
c70	2.1	F	+	727	1564	low grade lymphocytic infiltrate
c82	0.2	F	+	1014	736	cerebellar infarct & microhaemorrhages
c91	1.6	M	+	4047	1500	low grade lymphocytic infiltrate
c110	0.4	M	+	1134	1477	toxoplasmosis
c122	3.3	F	+	1932	2310	low grade lymphocytic infiltrate
c125	1.5	M	+	1236	885	no significant findings
c126	1.5	F	+	454	477	no significant findings
c129	0.4	F	+	925	1229	low grade lymphocytic infiltrate
c131	0.4	M	+	474	247	low grade encephalitis
c140	4.0	M	+	593	810	no significant findings
c150	0.4	F	+	716	661	nothing significant
c157	0.2	M	+	2715	1715	purulent meningitis
means				1355	987	
SD				1250	548	

CD68: The results are shown in Tables 4.3.4 and 4.3.5 but extracted here for easy reference as Tables 4.3.10 and 4.3.11, and summarised in Table 4.3.12 and illustrated in Figs 4.3.12- 4.3.18. In Tables 4.3.4 and 4.3.5, and in Tables 4.3.10 and 4.3.11, the HIV negative cases are grouped in the upper half, and HIV positive in the lower half. A simple way of considering the individual results is to divide the Table arbitrarily into 3 groups according to the CD68 values: **low** = 1 – 499, **medium** = 500 – 999, and **high** = 1,000 and above.

The means and standard deviations for the **HIV negative cases** in the basal ganglia grey matter were 542 (441) and **142(144)** in Groups A1 and B1 respectively, and those for the white matter, 443 (336) and **173 (160)** respectively (see Table 4.3.4). In the hippocampus, the corresponding means (Table 4.3.5) were in the medium range for Group A2, 796 (783) for grey matter and 686 (565) for white matter, and in the lower range for Group B2, **356 (333)** for grey matter and **304 (180)** for white matter. The difference between Group A1 and Group A2 means was due largely to the appreciably higher values for just two hippocampi (Group A2), which were not matched by high values in the 2 corresponding basal ganglia. The most striking results for individual HIV negative cases, in terms of high values in the basal ganglia, were cases **c33** (1,211 for grey matter and 1,088 for white) and **c77** (1,877 for grey matter and 1,231 for white) (Table 4.3.10). For both these cases, the pathology finding was possible septicaemia, a description based on the presence of large numbers of polymorphonuclear leucocytes in large and medium sized vessels. There was no recorded pathology within the brain itself in either of these cases. The possible significance of this finding is addressed in Chapter 6. Another case of possible septicaemia, **c101**, was associated with more modest values, 549 and 505 for

grey and white matter respectively, except in the hippocampus where the value for the white matter was 1406 (Table 4.3.11). In the basal ganglia there were 6 cases described as having no significant findings and for which the grey matter results fell in the range categorised as medium. In four of these the values were higher than those of the white matter. These cases, because of restricted block availability, did not have a matching hippocampus in Group A2. Nevertheless, 6 hippocampus cases had at least equivalent values for either grey or white matter, and three of these, **c60**, **c63** and **c124**, had either grey or white matter values in the high range. The two cases of cerebral malaria were both shared between the two sets, **c104** and **c117**. In the basal ganglia, the CD68 values were all in the low range except for that of the grey matter in case **c104**, 676. The values in the hippocampus were much higher, by a factor of two to three. There were 4 cases of purulent meningitis or meningoencephalitis and they were common to both sets. In two of these the results were in the low range, falling below the HIV negative means for both Groups A1 and 2, and B1 and 2. For the basal ganglia, these cases were **c59** and **c109**, and for the hippocampus, **c89** and **c109**.

In the **HIV positive cases,** strikingly higher CD68 values were seen, both in the basal ganglia and in the hippocampus (Tables 4.3.10 and 4.3.11). In 12 cases, the values for the grey matter were in the high range and for half of these, the corresponding white matter results were also high. The means (standard deviations) for Group A1, the basal ganglia, grey matter were 1038 (530) and for the white matter, 815 (476). The corresponding values for Group A2, the hippocampus, grey matter were 1355 (1250) and for the white matter, 987 (548). For Group BI, the basal ganglia, the values were 971 (533) for grey matter and 819 (601) for white matter.

For Group B2, the hippocampus, the values were 950 (537) for grey matter and 730 (163) for white matter

In the hippocampus, bearing in mind the slightly different case mix, there were slightly fewer grey matter values (n=9) and slightly more white matter values (n=8) in the high range than in the basal ganglia. Included in these (for both sets) were **c8**, a case with subtle widespread white matter damage, and **c24**, the case of measles encephalitis. The AIDS case with toxoplasmosis, **c110**, which was common to both, showed high values in the basal ganglia and in the grey matter of the hippocampus, with medium values for hippocampal white matter. The other AIDS case, **c152**, with HIVE and toxoplasmosis, was part of the basal ganglia set only. Neither the value for grey matter, 741, nor that for white matter, 348, fell in the high range.

There were 7 cases described as showing low grade lymphocytic infiltrate in each set. Of those in the basal ganglia collection, four had grey matter values in the high range while there were two high values in the hippocampus subset. For one case in each group, both grey and white matter values were in the low range. There was only one case of meningitis shared between the basal ganglia and hippocampus sets, **c157**, and in both sets it was associated with high values except for the white matter in the basal ganglia, where a value of 922 was obtained. The grey and white matter values of three HIV positive cases in the basal ganglia group were below the means for the HIV negative group. These 3 cases were **c121**, with cerebral oedema, as well as **c126** and **c140**, both with no significant findings. In the hippocampus group, **c126**, with no significant findings, had values which approximated to the means and one case, **c131**, with low grade encephalitis, had values which were lower.

The results of Mann-Whitney tests for CD68 immunostaining in the HIV negative and positive basal ganglia and hippocampus are shown in Table 4.3.12. It can be seen that for the basal ganglia, the activation was greater in the grey matter than the white, for both HIV negative and positive groups, and that the differences between HIV positive and negative grey and white matter in Group A1 are highly significant (p=0.001 and p=0.000, respectively). Even in the small subset, Group B1, the basal ganglia grey matter of HIV positive cases, though not the white matter, showed significantly more CD68 positivity than did the HIV negative cases (p=0.021). The same pattern was true for the hippocampus but a statistically significant increase in HIV positive cases was seen only in white matter in Group B2 (p=0.022).

The CD68 results are also displayed in the boxplots, Figs 4.3.27, 4.3.28, 4.3.37 and 4.3.38.

Table 4.3.12 Mann-Whitney test results for CD68 image analysis: basal ganglia and hippocampus.

CD68 image analysis results				**CD68 image analysis results**			
Basal ganglia - Mann-Whitney tests				**Hippocampus - Mann-Whitney tests**			
		gm medians	wm medians			gm medians	wm medians
Group A1	HIV -ve	380	373	Group A2	HIV -ve	697	458
	HIV +ve	1063	761		HIV +ve	826	837
	p value	**p=0.002**	**p=0.009**		p value	**p=0.091**	**p=0.468**
Group B1	HIV -ve	364	383	Group B2	HIV -ve	157	244
	HIV +ve	1076	698		HIV +ve	716	810
	p value	**p=0.142**	**p=0.403**		p value	**p=0.144**	**p=0.022**

HLA: the pattern obtained with anti-HLA antibody was almost identical to that obtained with anti-CD 68 and no quantitative analysis has been attempted.

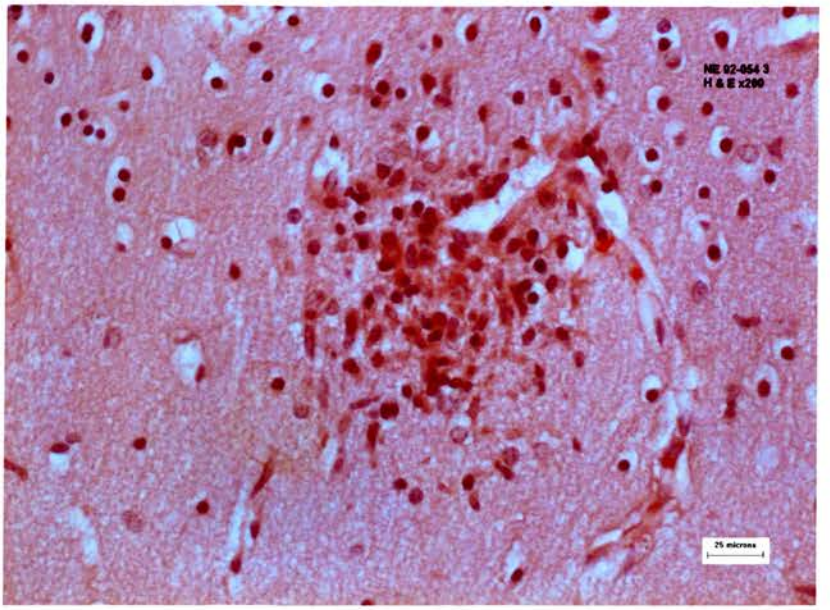
Summary of Immunohistochemical Results

Comparison of the CD16 results in Tables 4.3.5 and 4.3.5 with those for CD 68 shows some notable parallel results. For example, the high levels of CD68 immunostaining for **c8** and **c157** were seen to a lesser degree for CD16. This was also the case for the low grade lymphocytic infiltrate case, **c122**. Similarly high CD16 and CD68 values were seen in the cases with possible septicaemia and the same was true for **c54,** with meningoencephalitis. Some equally marked differences were also seen. For example, the very high CD68 values in the measles encephalitis case, **c24**, were not reproduced in the CD16 values. Of the malaria cases, **c104** had the highest CD68 values in the hippocampus, whereas for CD16 it was **c117**.

The results of all the Mann-Whitney tests for the immunostains for lymphocytes and microglia/macrophages in HIV positive and negative cases, and in the basal ganglia and hippocampus, are summarised in Appendix **17** that combines the data shown in Tables 4.3.6, 4.3.7, 4.3.8, 4.3.9 and 4.3.12.

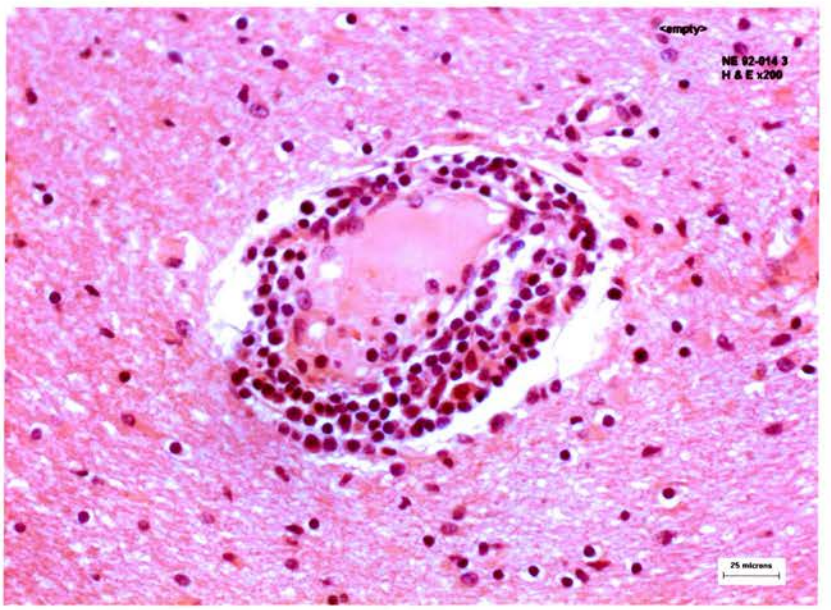
For the Figures included below, **BG** denotes basal ganglia and **HC** denotes hippocampus

Fig. 4.3.1 Hippocampus – Haematoxylin & Eosin x200



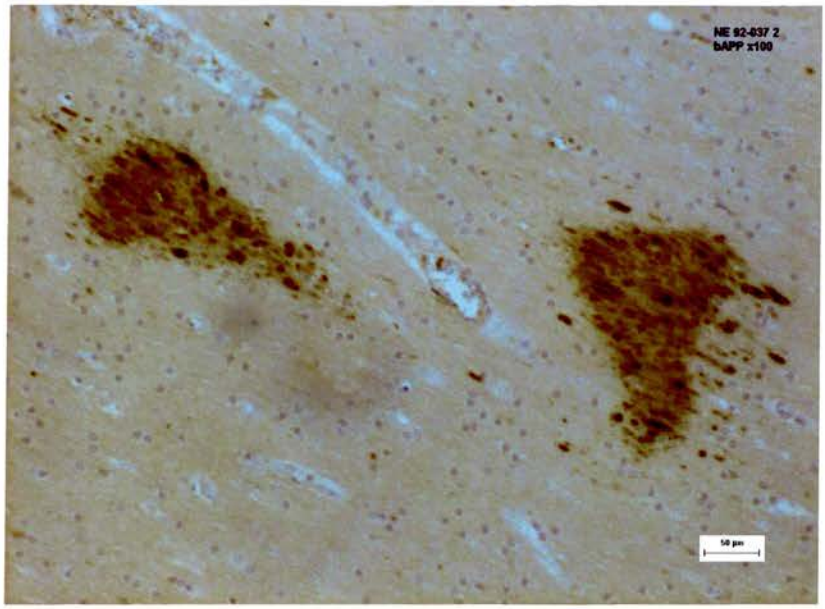
Notes: Case c110 – an HIV positive case with AIDS showing a perivascular focus of toxoplasmosis.

Fig. 4.3.2 Hippocampus – Haematoxylin & Eosin x200



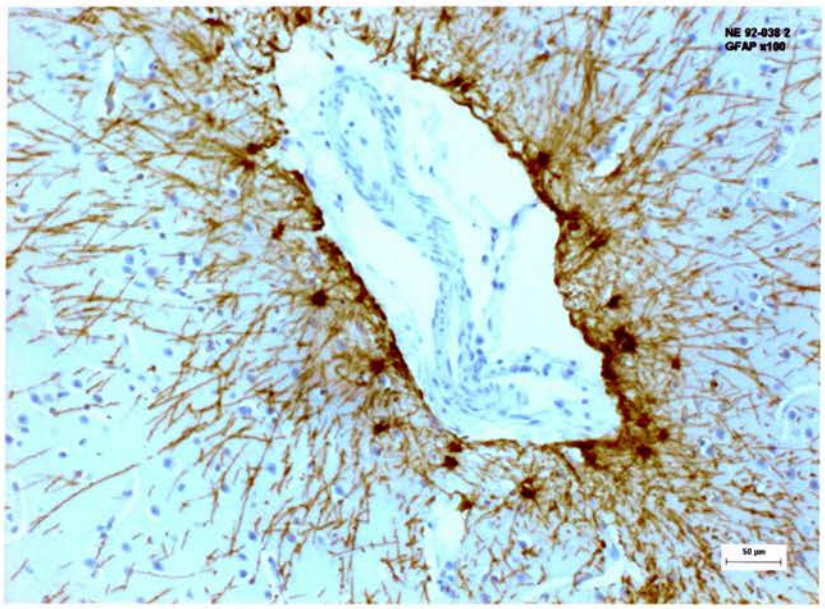
Notes: Case c70 – an HIVpositive case showing a pleomorphic perivascular cuff. One of the cases listed as "low-grade lymphocytic infiltrate".

Fig. 4.3.3 Basal Ganglia White Matter – βAPP x100



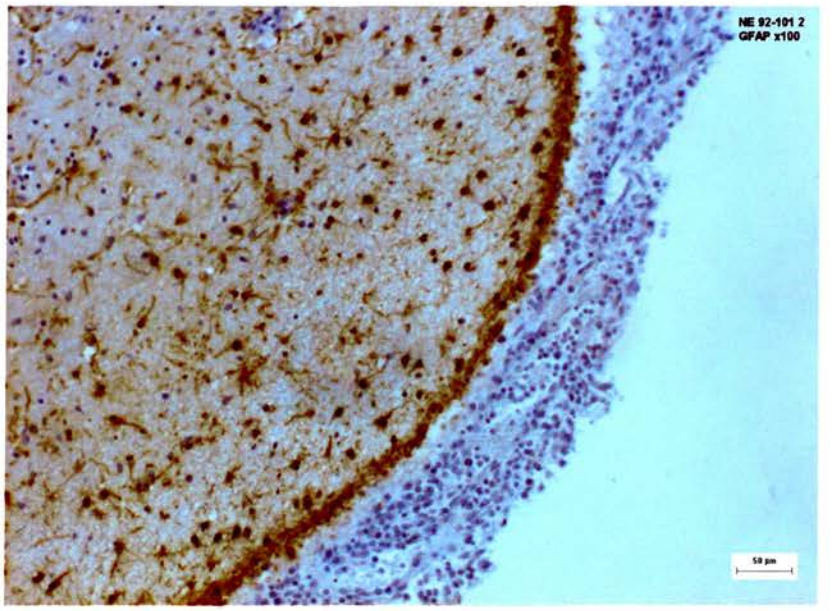
Notes: Case c93 – an HIV negative case showing evidence of axonal damage. A case reported as "no significant findings" on routine screening.

Fig. 4.3.4 Basal Ganglia – White Matter – GFAP x100



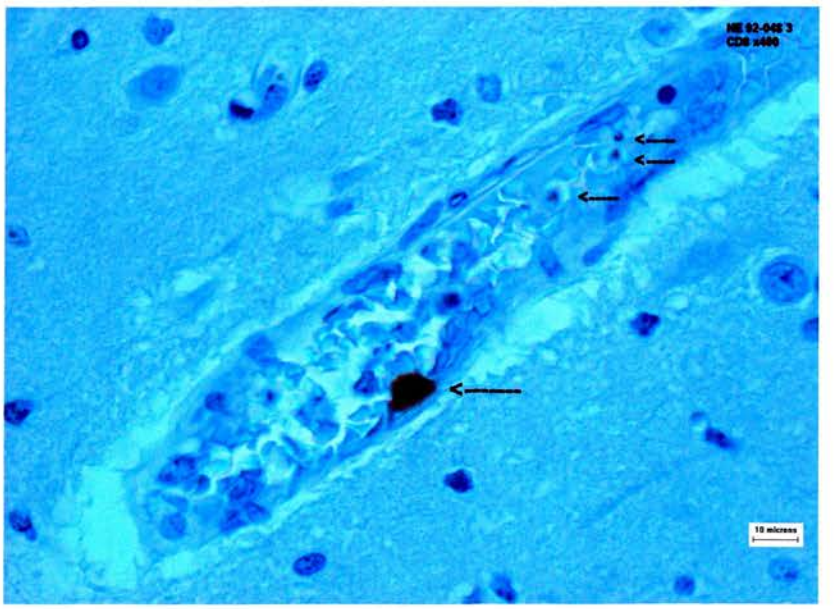
Notes: Case c94 – an HIV negative case described as "no significant findings" on routine screening and showing increased perivascular GFAP expression

Fig. 4.3.5 Basal Ganglia - Ependymal Surface – GFAP x100



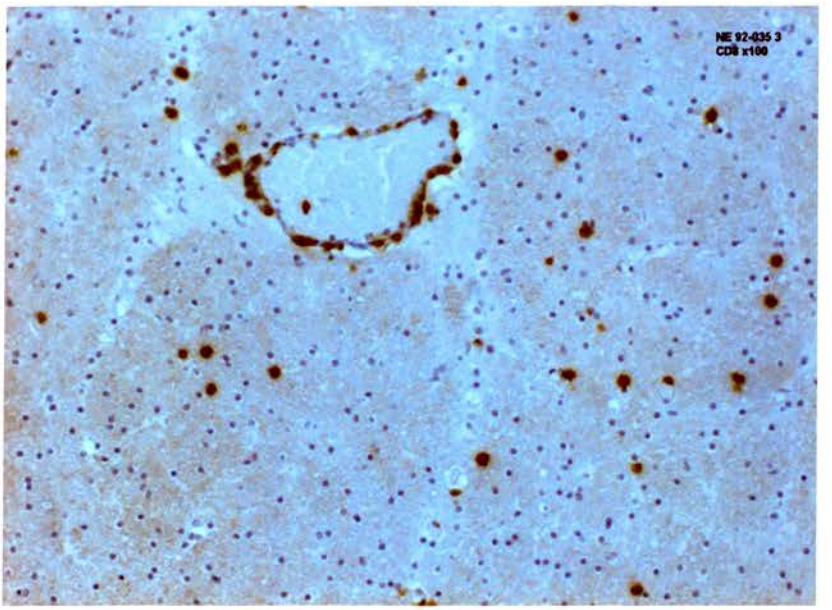
Notes: Case c157 - an HIV positive case with purulent meningitis and showing a diffuse increase of GFAP expression in the caudate nucleus.

Fig. 4.3.6 Hippocampus – Grey Matter – CD8 x400



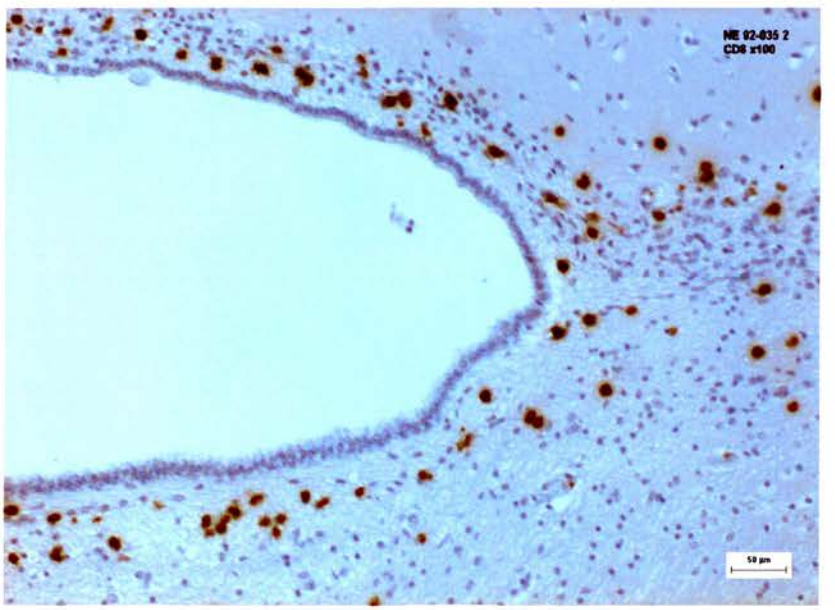
Notes: Case c104 - an HIV negative case with cerebral malaria. A longitudinal section of a vessel showing congestion and a CD8 positive lymphocyte adherent to the endothelium – long arrow, and malarial pigment in red cells – shorter arrows.

Fig. 4.3.7 Basal Ganglia – Internal Capsule – CD8 x100



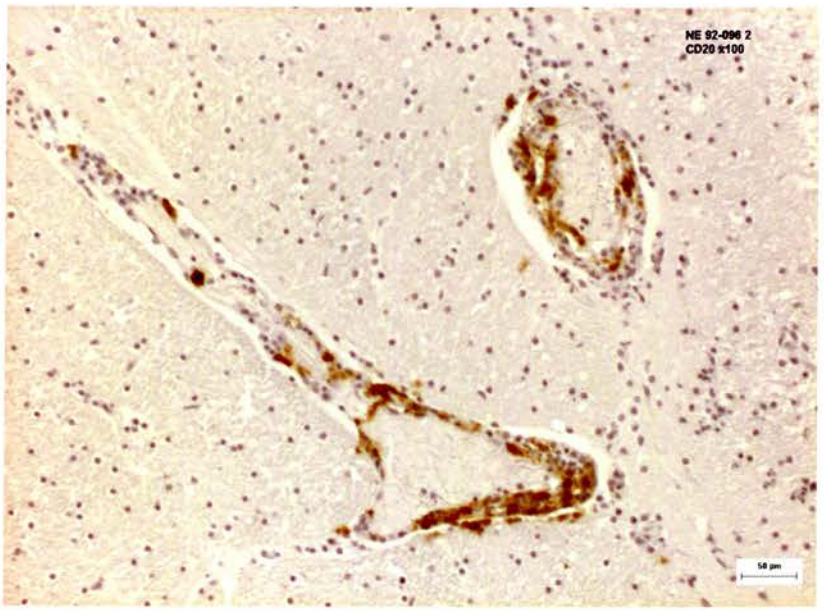
Notes: Case c91 – an HIV positive case, " low grade lymphocytic infiltration" showing CD8 positive perivascular and parenchymal cells.

Fig. 4.3.8 Basal Ganglia – Lateral Angle of Ventricle – CD8 x100



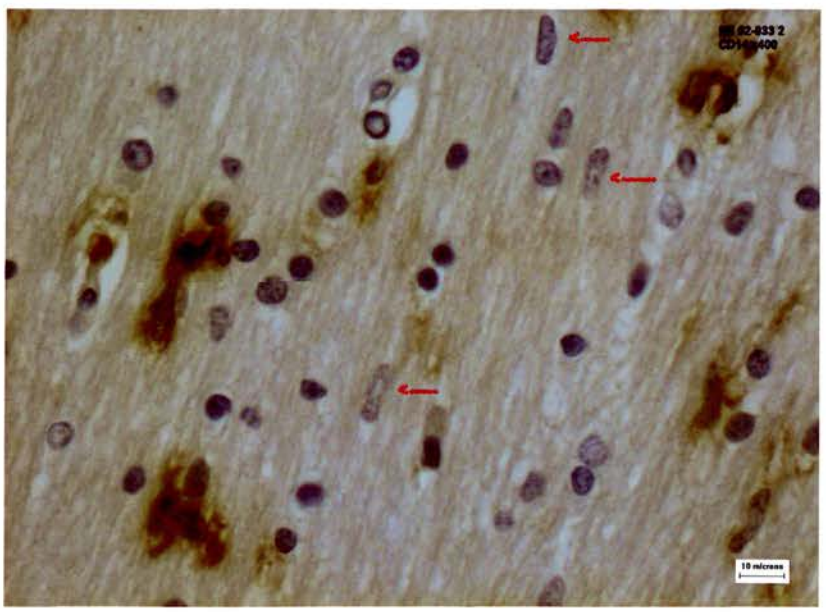
Notes: Case c91 – the same case as above showing numerous CD8 positive cells in subependymal layer.

Fig. 4.3.9 Basal Ganglia – White Matter - CD20 x100



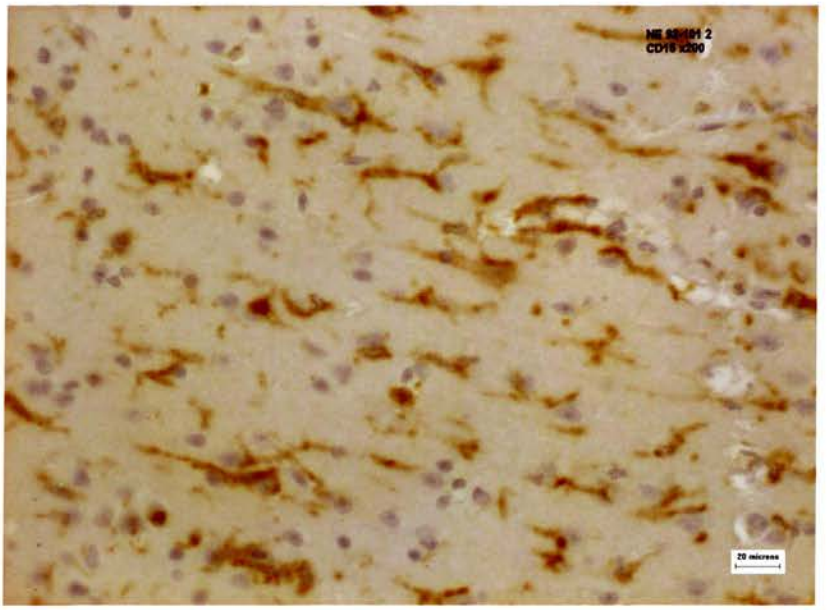
Notes: Case c152 – HIV encephalitis and toxoplasmosis showing numerous perivascular CD20 cells.

Fig. 4.3.10 Basal Ganglia – White Matter – CD14 x400



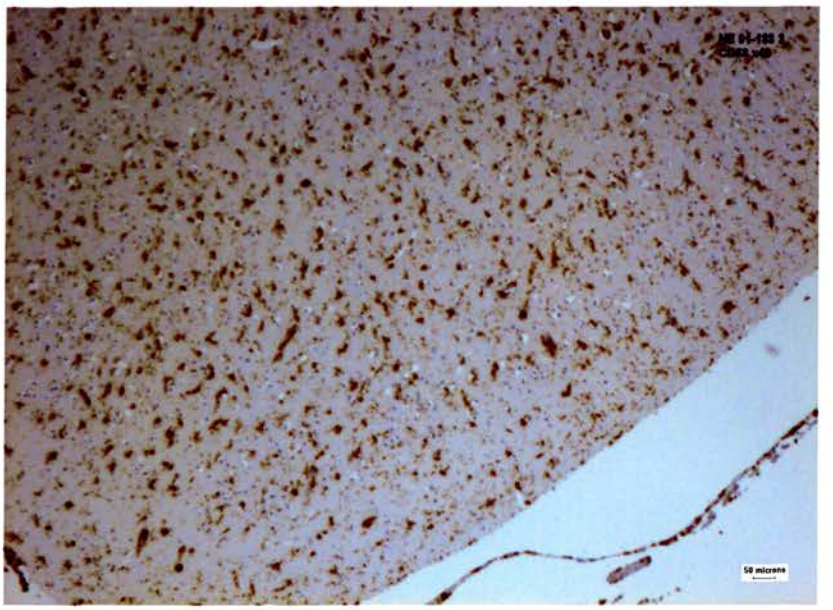
Notes: Case c89 – an HIV negative case with meningitis showing CD14 positive perivascular cells and CD14 negative macrophages/microglia – red arrows. Note the difficulty in identifying individual stained cells (see note in first paragraph of materials and methods)

Fig. 4.3.11 Basal Ganglia - Grey Matter – CD16 x200



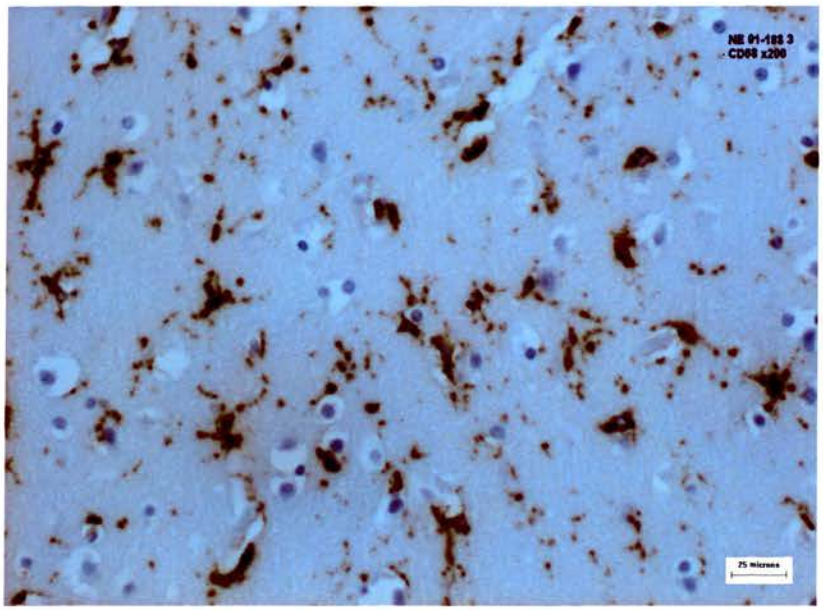
Notes: Case c157 – an HIV positive case with meningitis - grey matter showing diffuse CD16 positive macrophage/microglial expression.

Fig. 4.3.12 Hippocampus – Cortex – CD68 x40



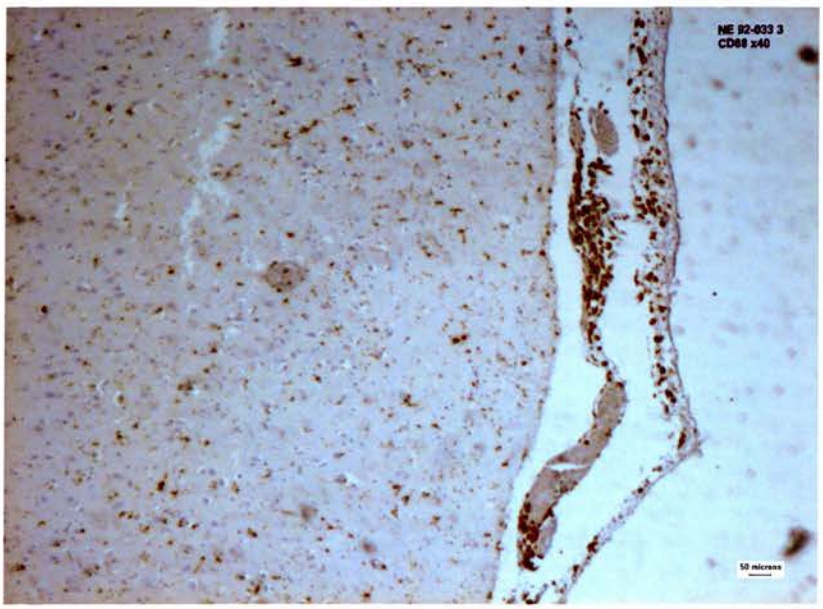
Notes: Case c33 – an HIV negative case with possible septicaemia showing dense diffuse CD68 expression.

Fig. 4.3.13 Hippocampus – Cortex – CD68 x200



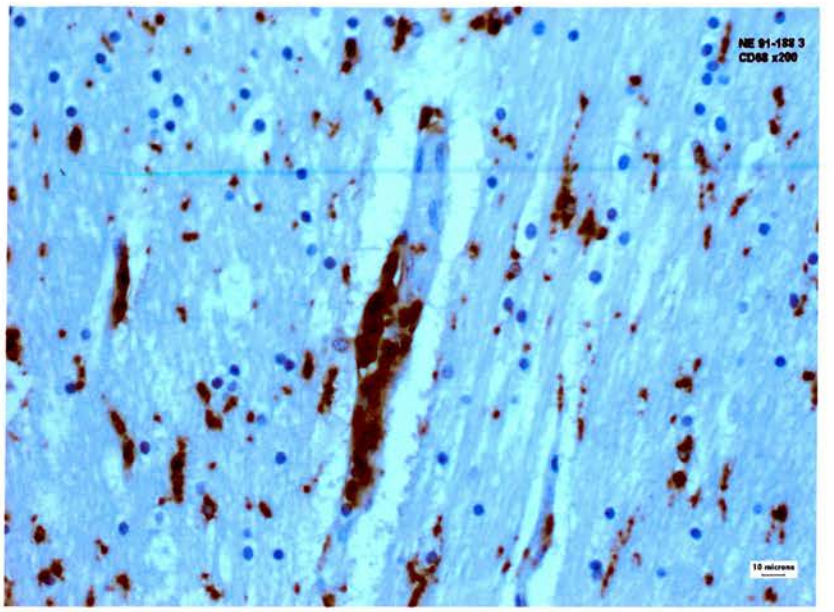
Notes: Case c33 – same case as above at higher magnification

Fig. 4.3.14 Hippocampus – Cortex – CD68 x40



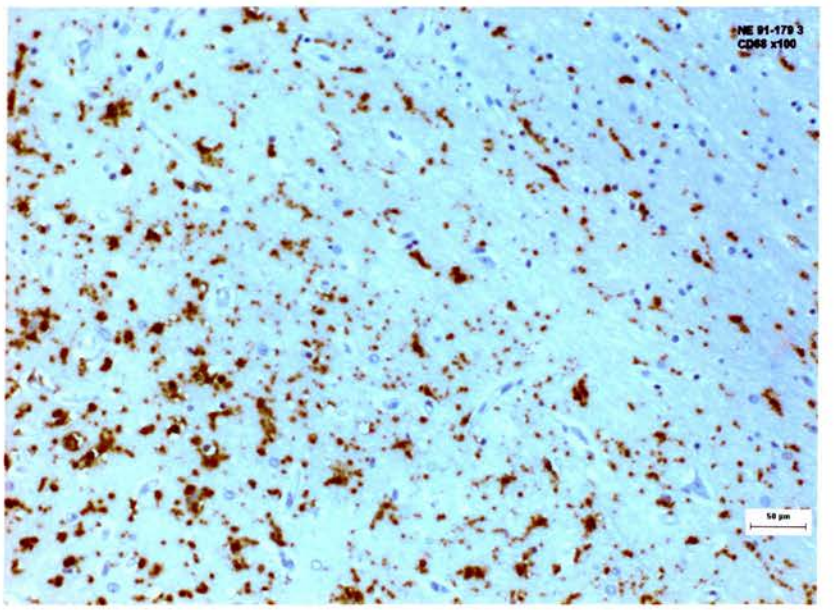
Notes: Case c89 – An HIV negative case with purulent meningitis showing relatively little CD68 expression in the cortex.

Fig. 4.3.15 Hippocampus – White Matter – CD68 x200



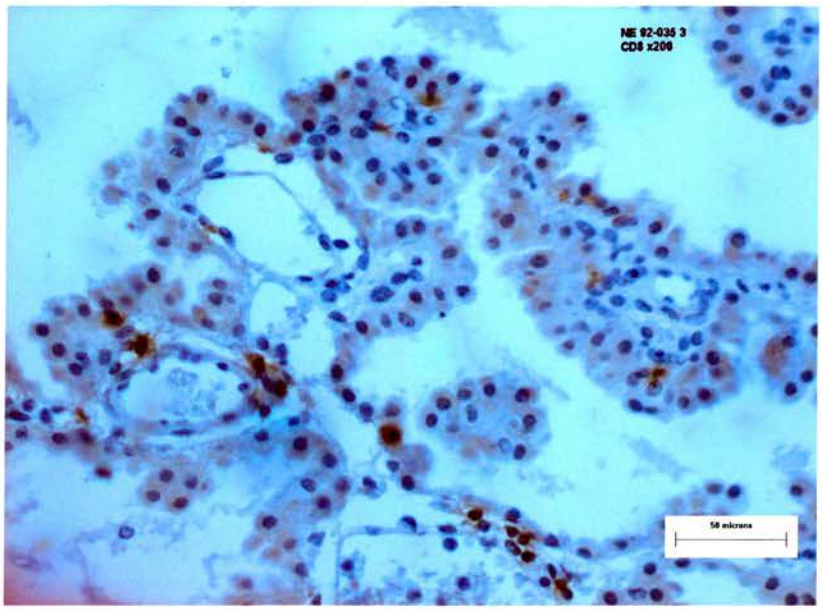
Notes: Case c33 – an HIV negative case with septicaemia showing CD68 positive cells around a small vessel in longitudinal section.

Fig. 4.3.16 Hippocampus – Grey Matter/White Matter Junction CD68 x100



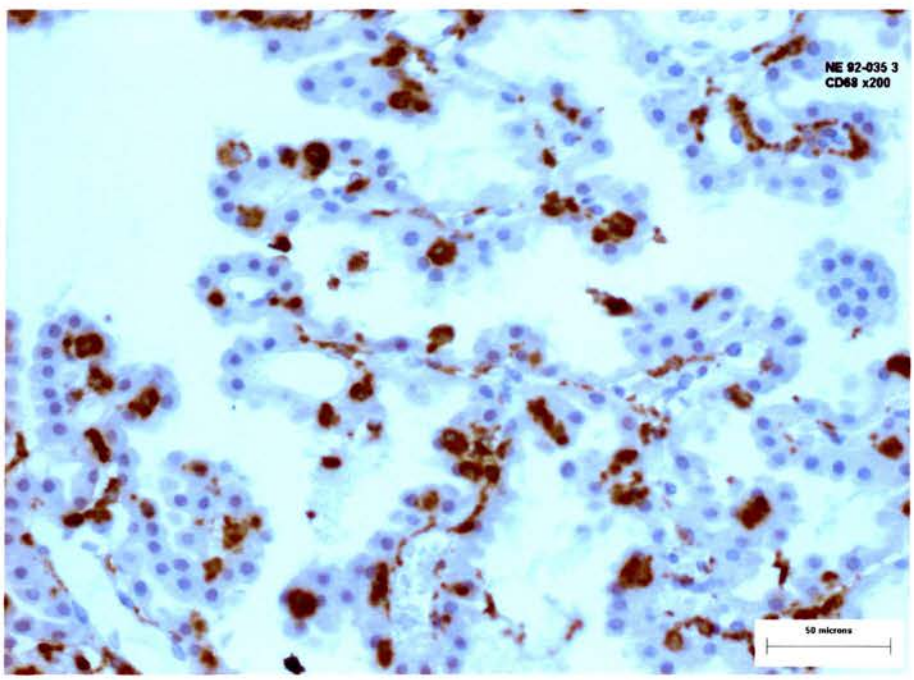
Notes: Case c24 – an HIV negative case with measles encephalitis - CD68 immunostain showing the differing expression between grey matter bottom left and white matter top right.

Fig. 4.3.17 - Hippocampus – Choroid Plexus – CD8 x200



Notes: Case c91 – an HIV positive case with low grade lymphocytic Infiltrate - to show CD8 positive cells in choroid plexus.

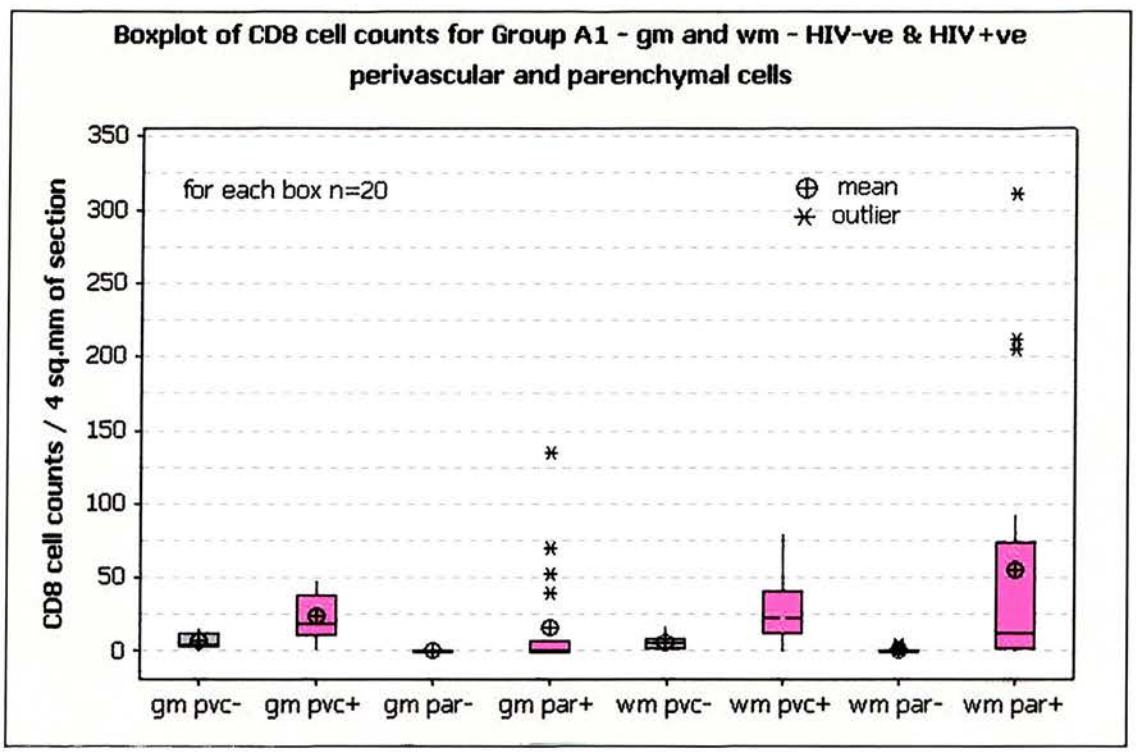
Fig. 4.3.18 - Hippocampus – Choroid Plexus – CD68 x200



Notes: Case c91 – the same case as above to show CD68 positive cells in choroid plexus.

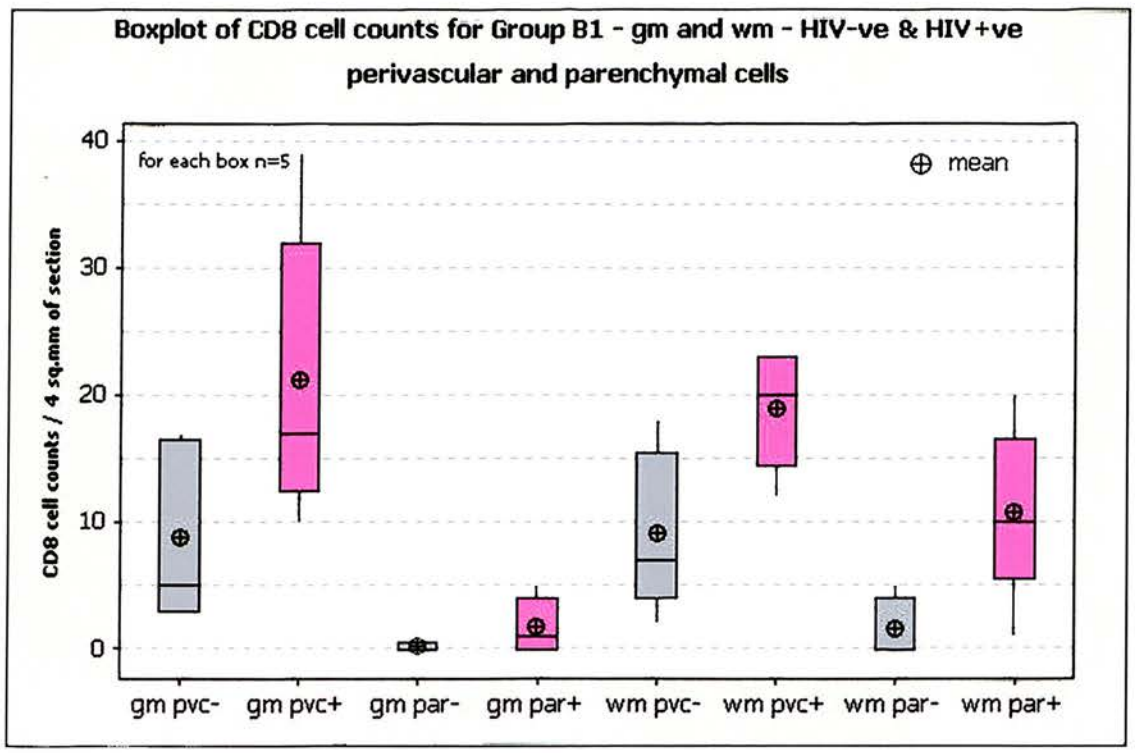
In the following charts the basal ganglia are denoted by "BG" or "bg" and hippocampus by "HC" or "hc". Grey matter by "gm" and white matter by "wm". Boxes for HIV positive cases have a pink background.

Fig. 4.3.19 BG CD8 - Group A1



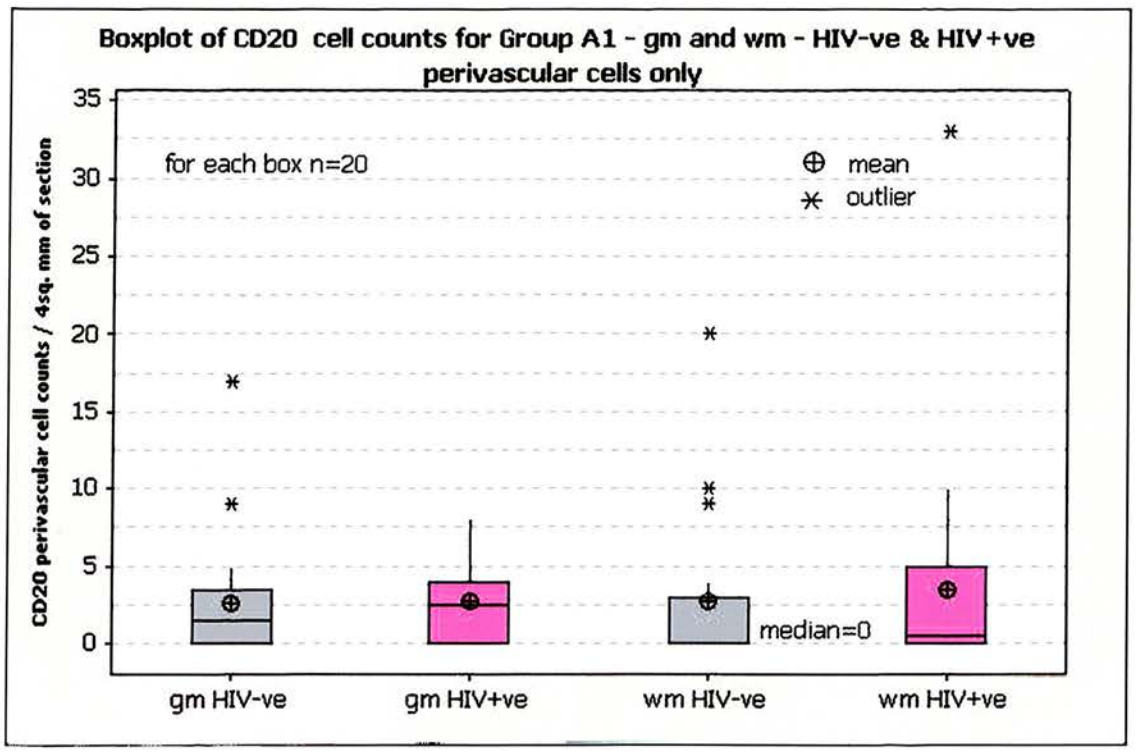
Boxplot of counts for perivascular and parenchymal cells in caudate nucleus (gm) and internal capsule (wm)

Fig. 4.3.20 BG CD8 Group B1



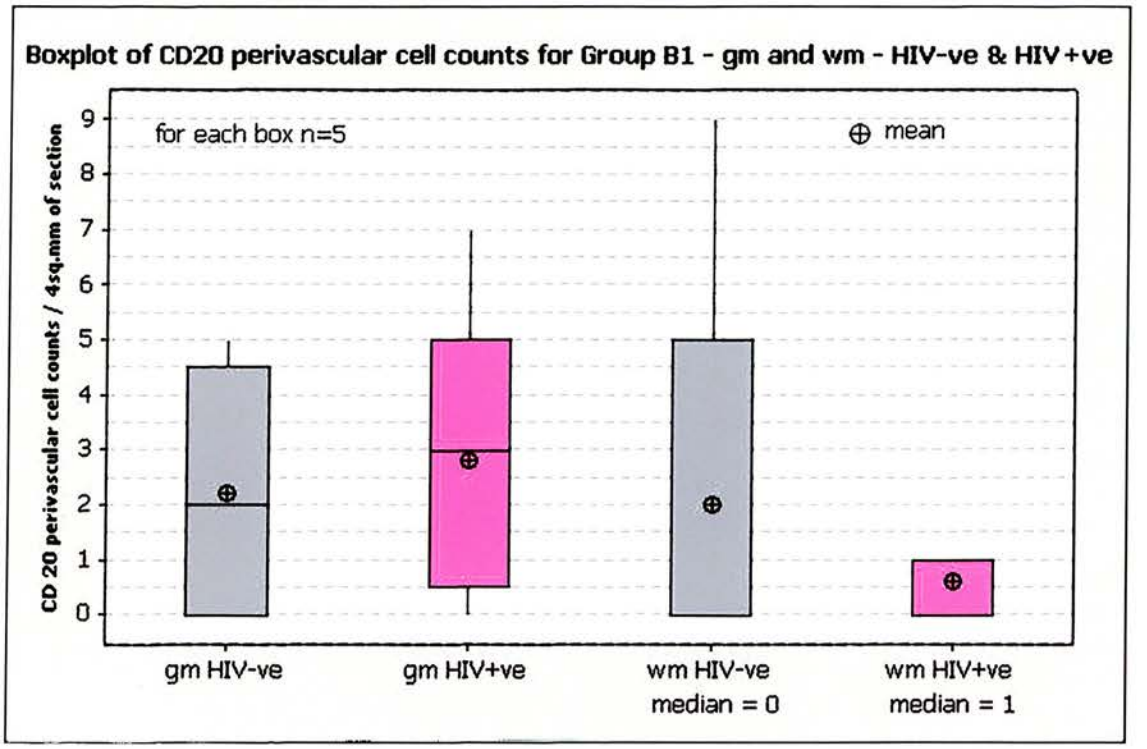
Boxplot of counts for perivascular and parenchymal cells in caudate nucleus (gm) and internal capsule (wm)

Fig. 4.3.21 - BG - CD20 Group A1



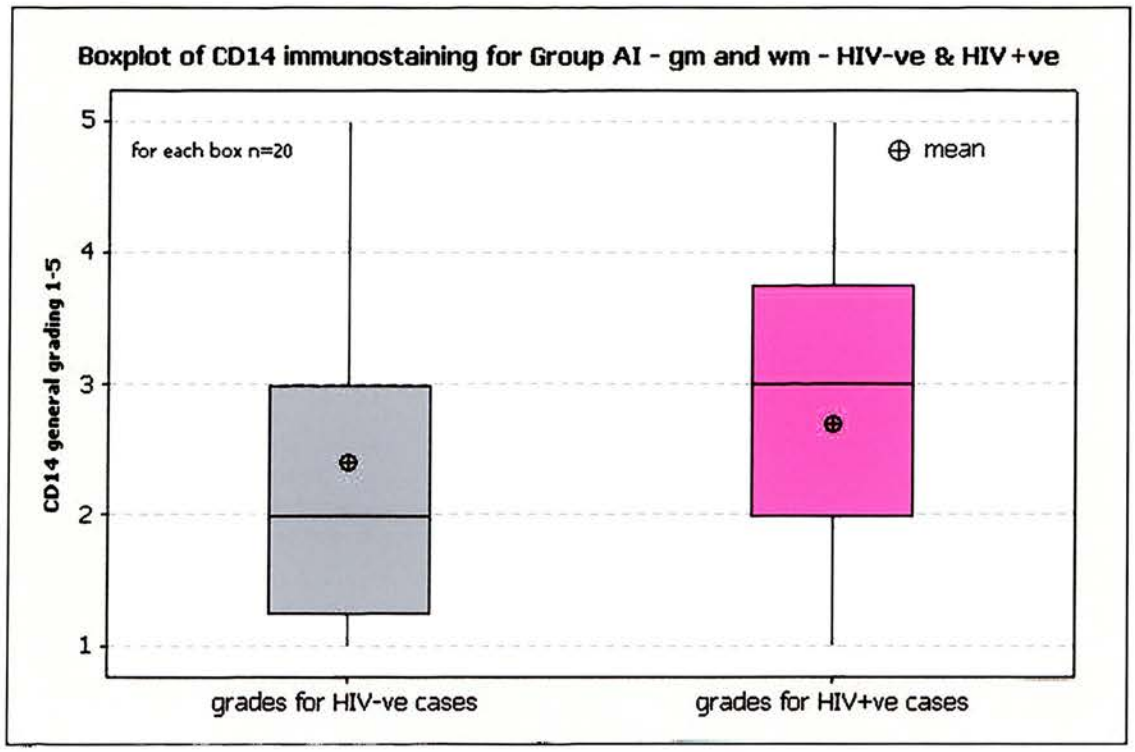
Boxplot of perivascular cell counts only

Fig. 4.3.22 BG CD20 Group B1



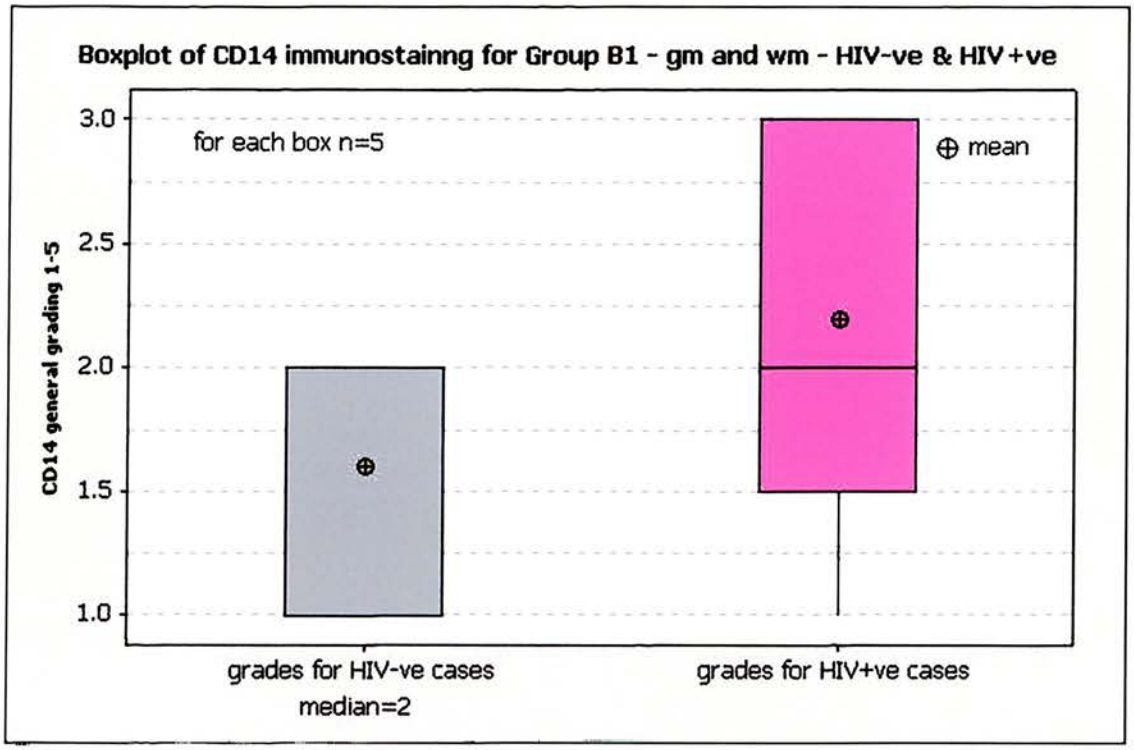
Boxplot of perivascular cell counts only

Fig. 4.3.23 BG CD14 Group A1



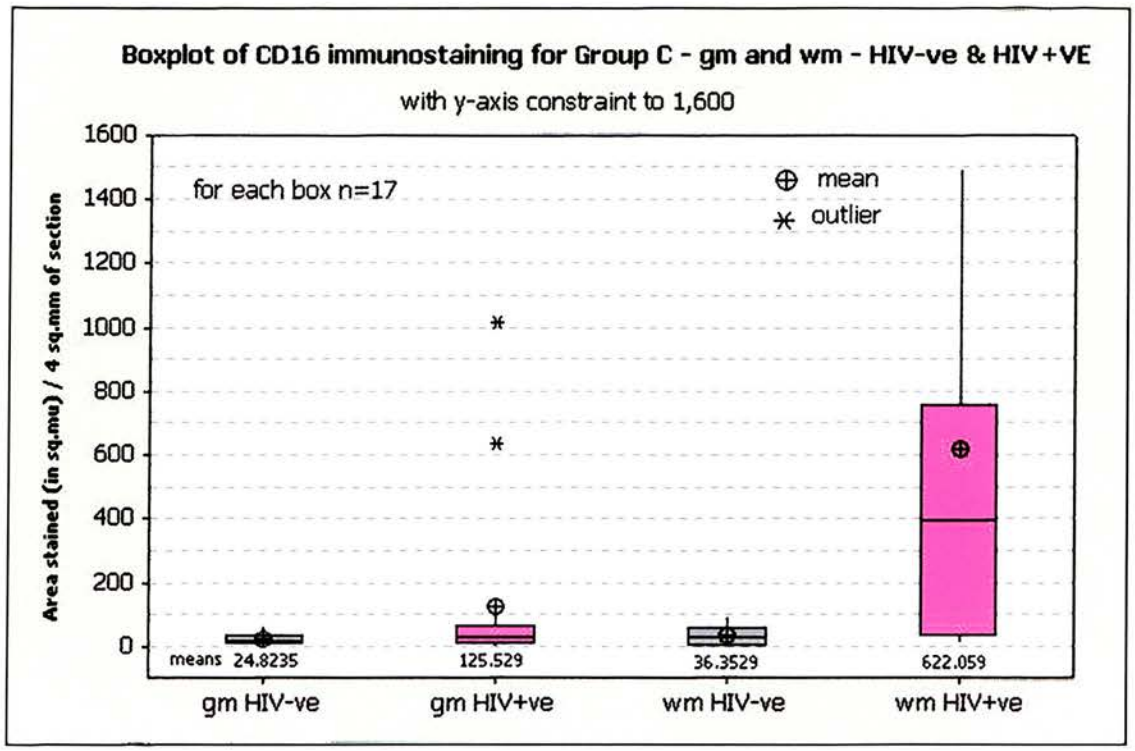
Boxplot of general (gm and wm) grades 1 -5

Fig 4.3.24 – BGCD14 Group B1



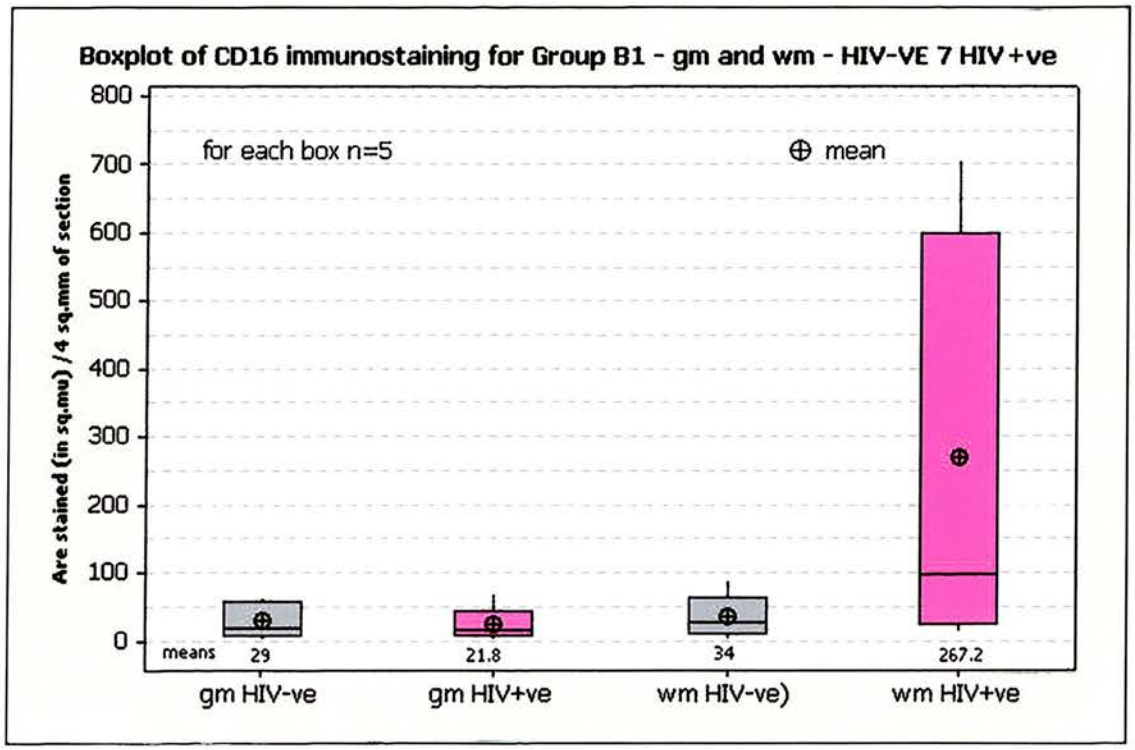
Boxplot of general (gm and wm) grades 1-5

Fig 4.3.25 BG CD16 Group C



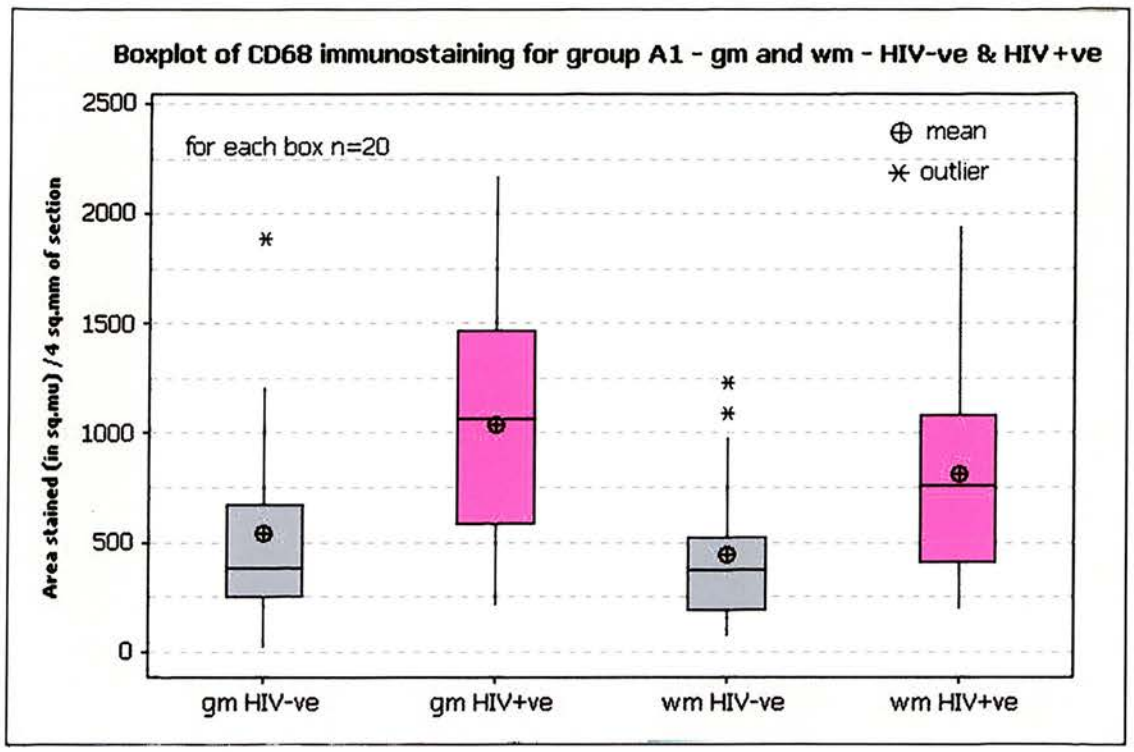
Boxplot of image analysis for caudate nucleus (gm) and internal capsule (wm); graph is with y-axis constraint of 1,600

Fig. 4.3.26 BG CD16 Group B1



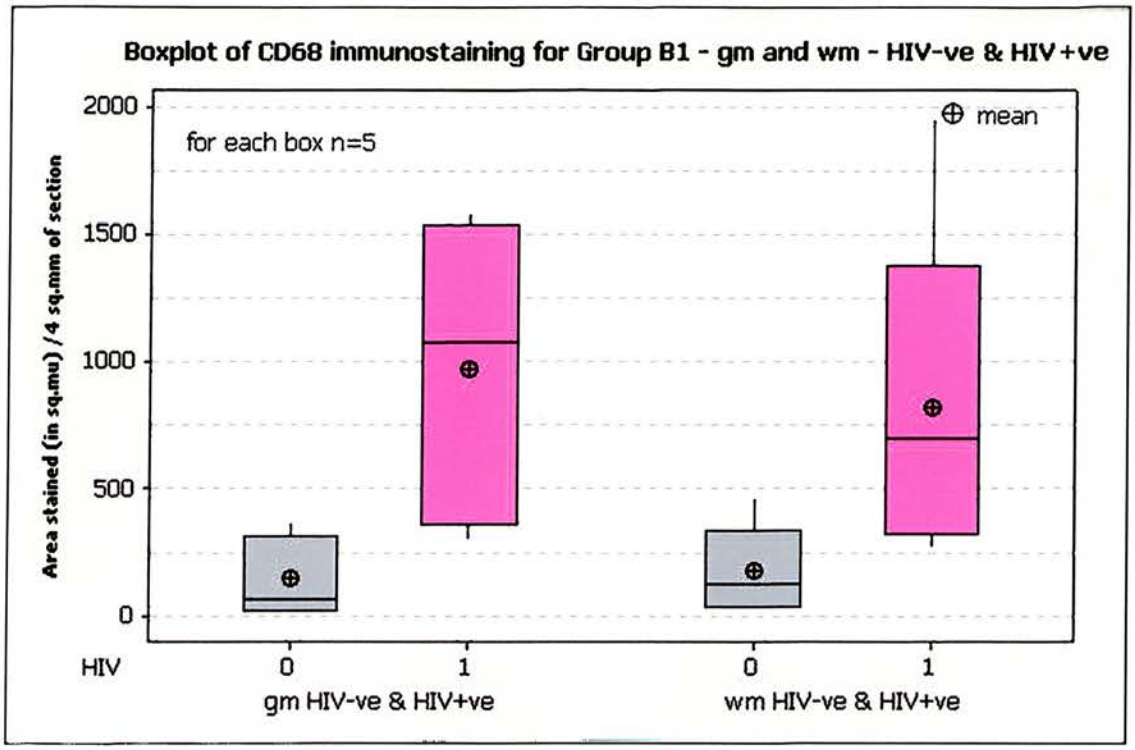
Boxplot of image analysis for caudate nucleus (gm) and internal capsule (wm)

Fig. 4.3.27 BG CD68 Group A1



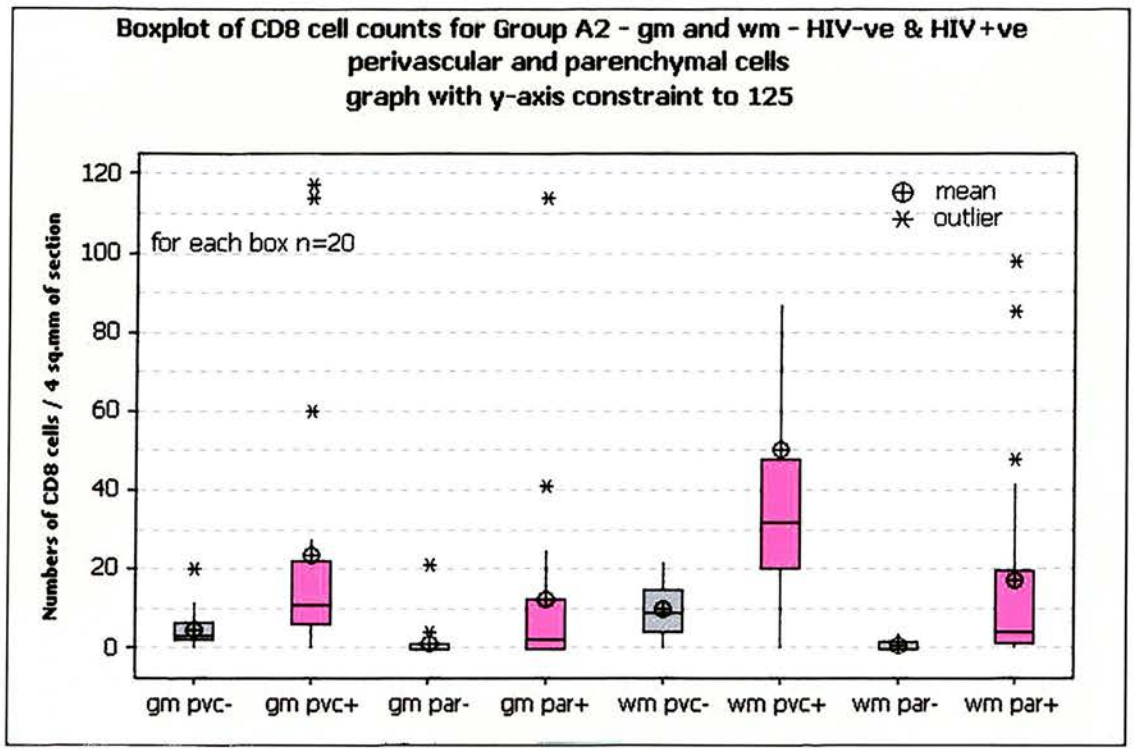
Boxplot of image analysis for caudate nucleus (gm) and internal capsule (wm)

Fig. 4.3.28 BG CD68 Group B1



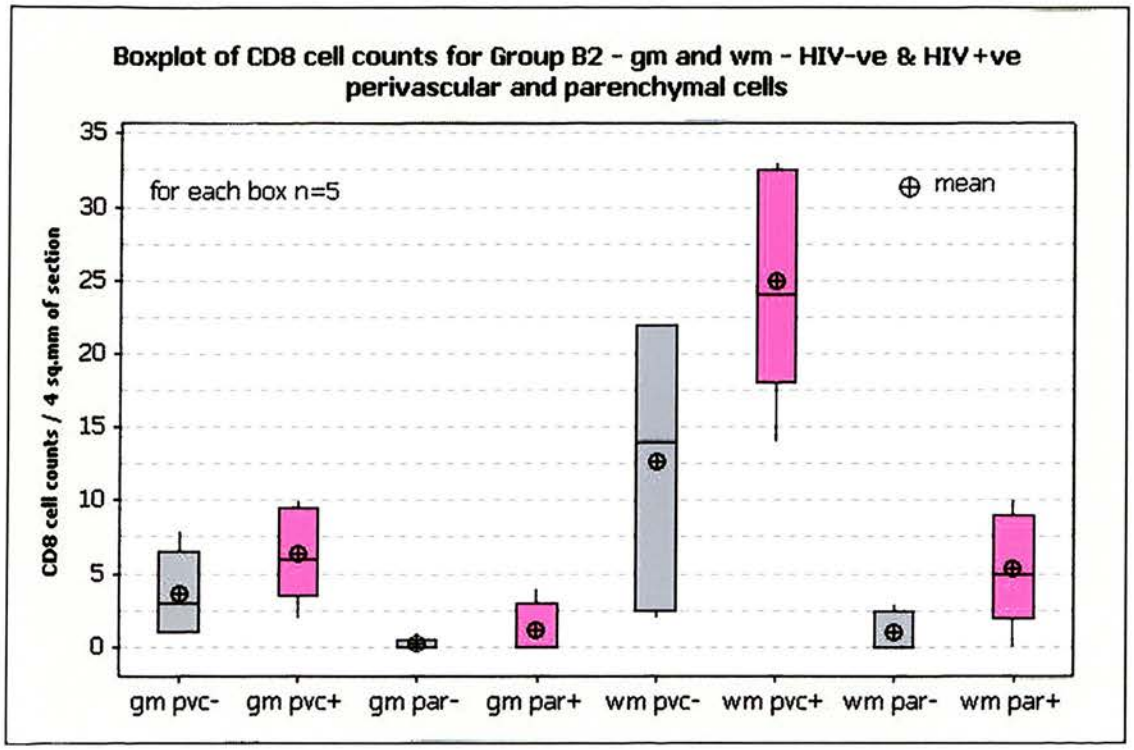
Boxplot of image analysis for caudate nucleus (gm) and internal capsule (wm)

Fig. 4.3.29 HC CD8 Group A2



Boxplot of perivascular and parenchymal cell counts

Fig. 4.3.30 HC CD8 Group B2



Boxplot of perivascular and parenchymal cell counts

Fig. 4.3.31 HC CD20 Group A2 – boxplot of perivascular cell counts only

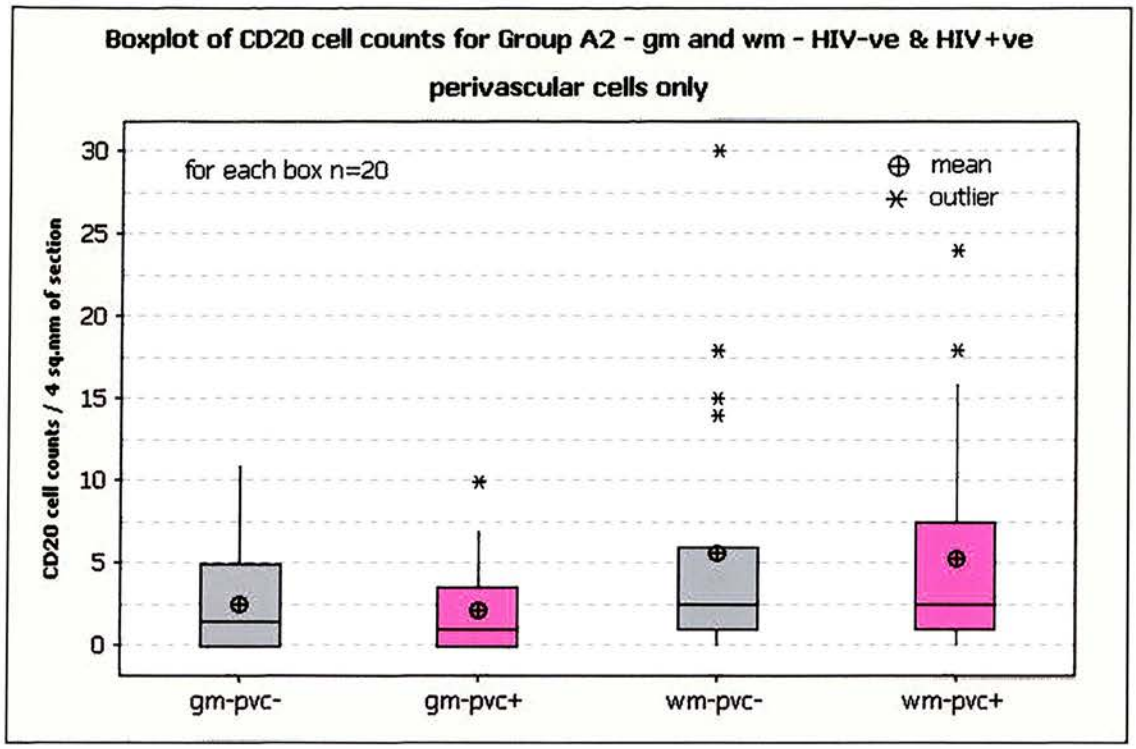


Fig. 4.2.32 HC CD20 Group B2 - perivascular cell counts only

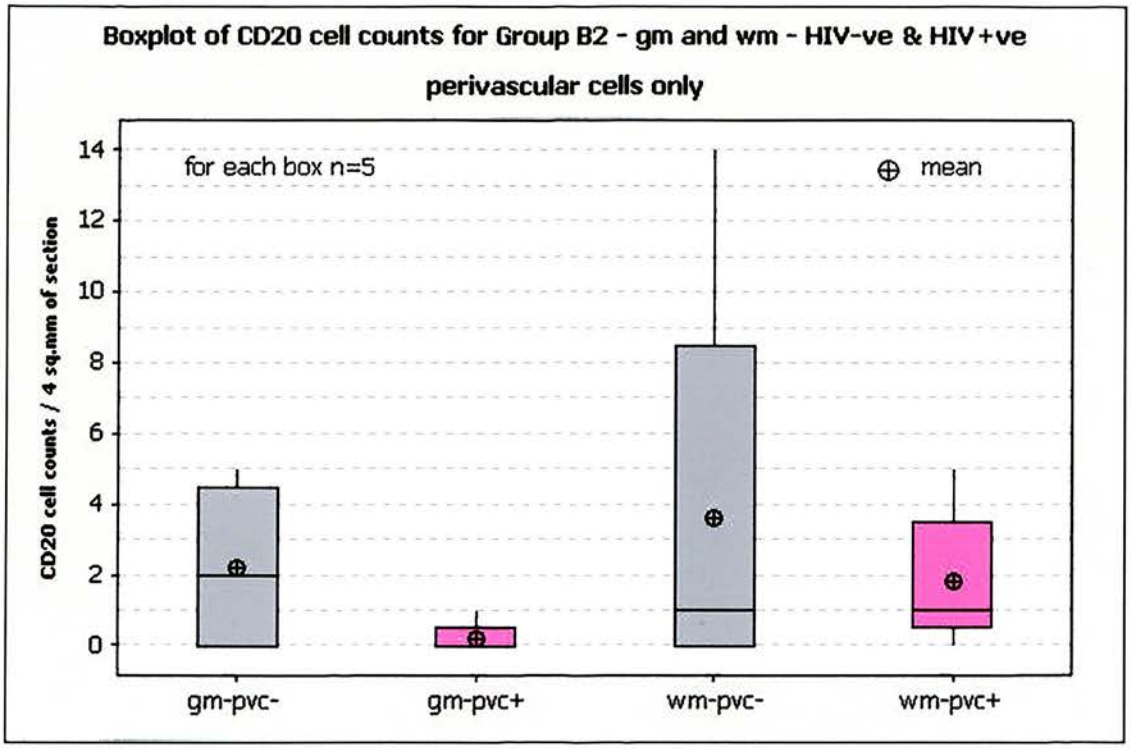


Fig. 4.3.33 HC CD14 Group A2 – boxplot of general grades 1-5

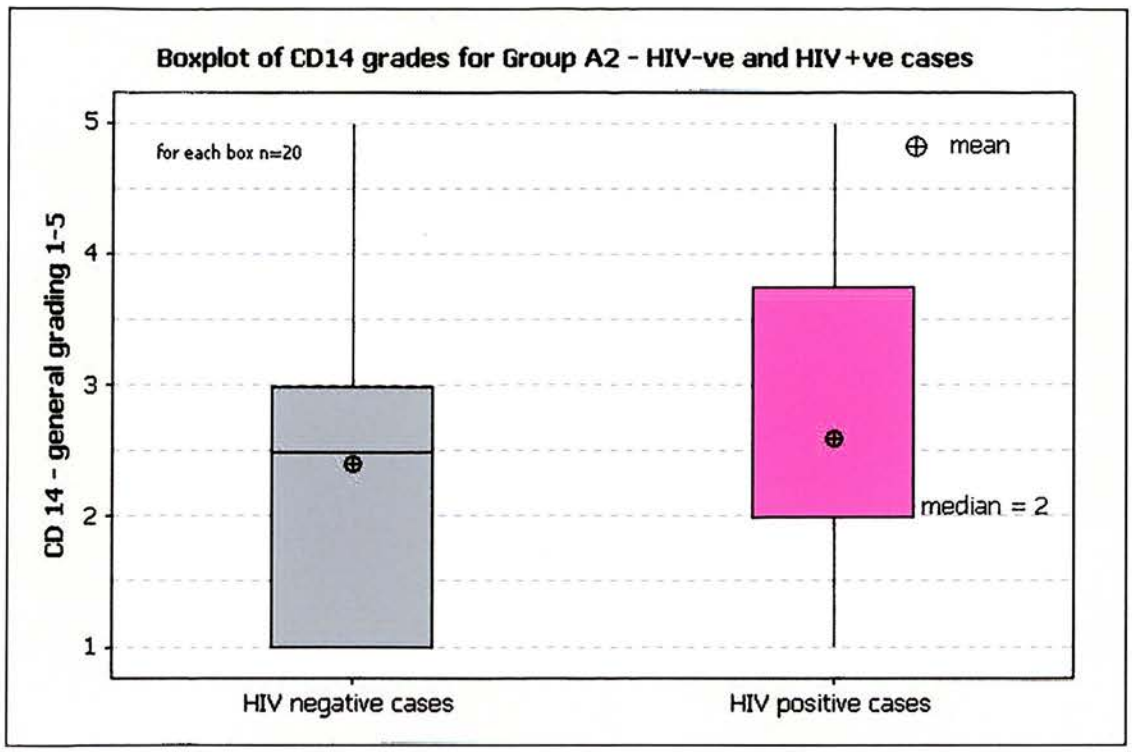


Fig. 4.3.34 HC CD14 Group B2 – boxplot of general grades 1-5

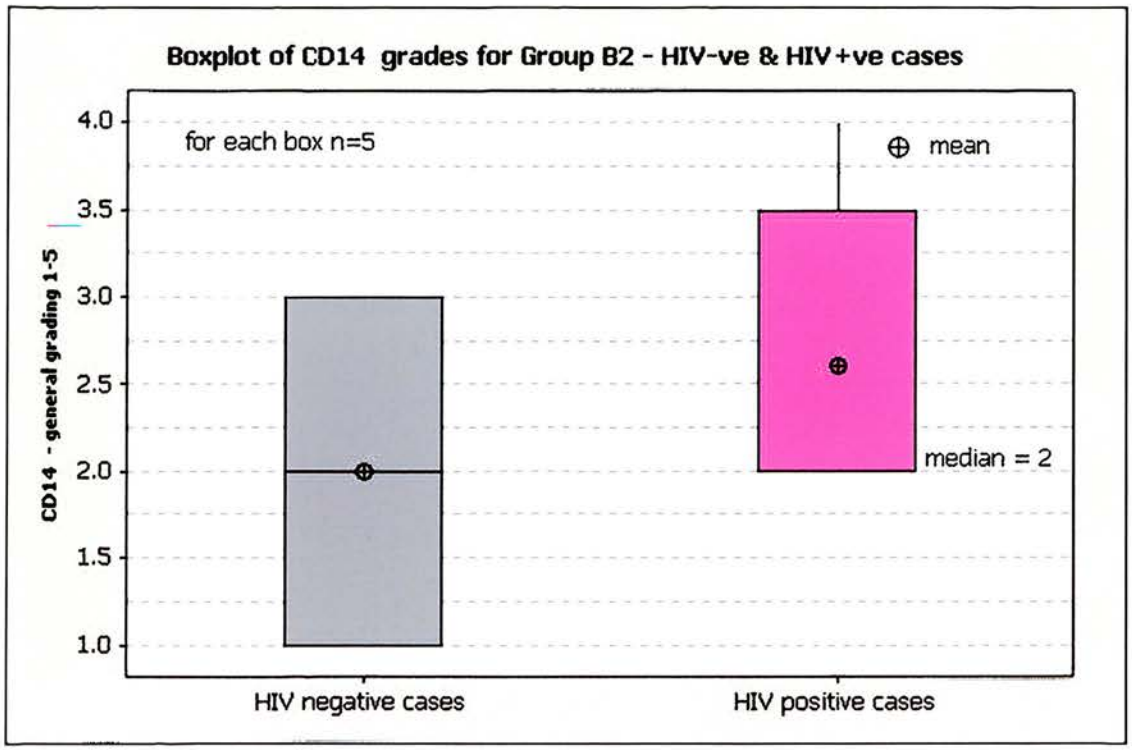


Fig. 4.3.35 - HC CD16 Group A2 - boxplot of image analysis for grey matter and white matter; graph has y-axis constraint to 1,500

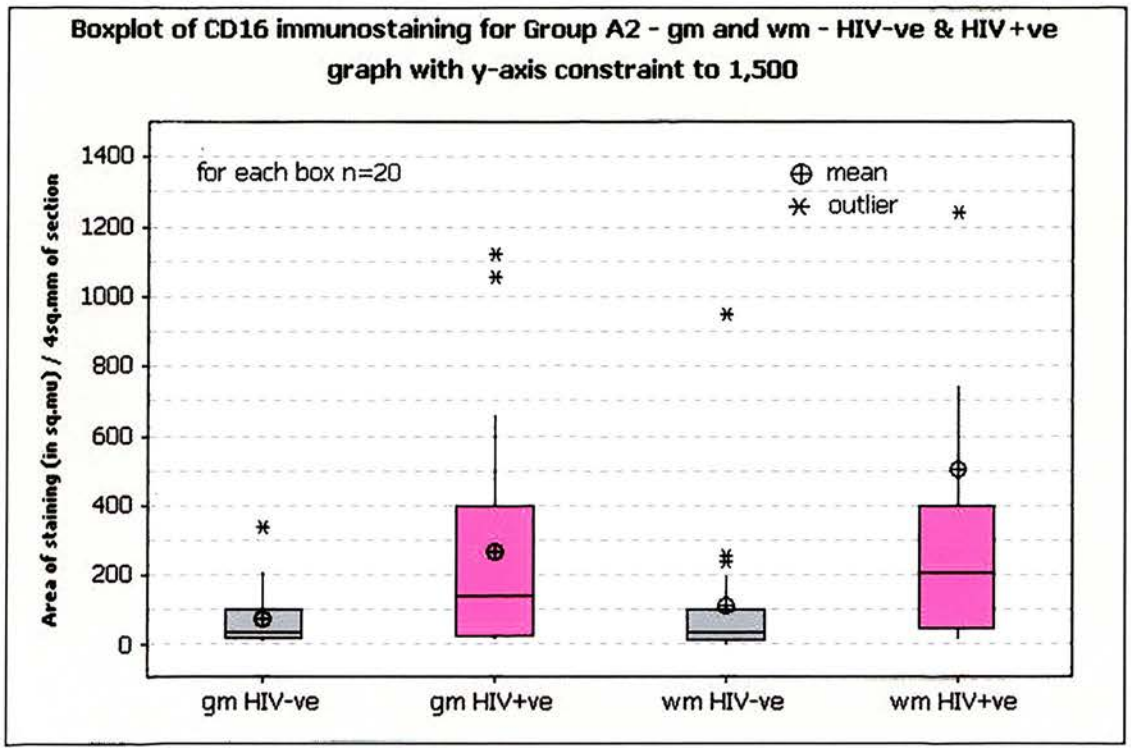


Fig. 4.3.36 HC CD16 Group B2 – boxplot of image analysis for grey and white matter

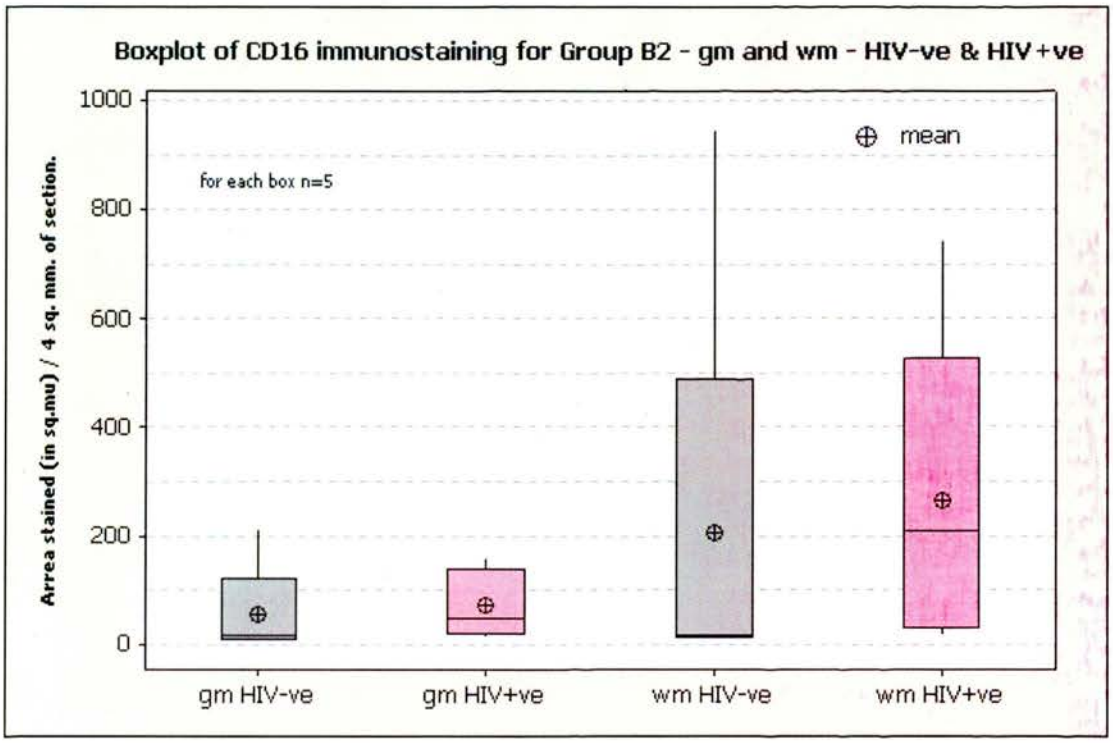


Fig. 4.3.37 HC CD68 Group A2 – boxplot of image analysis for grey matter and white matter

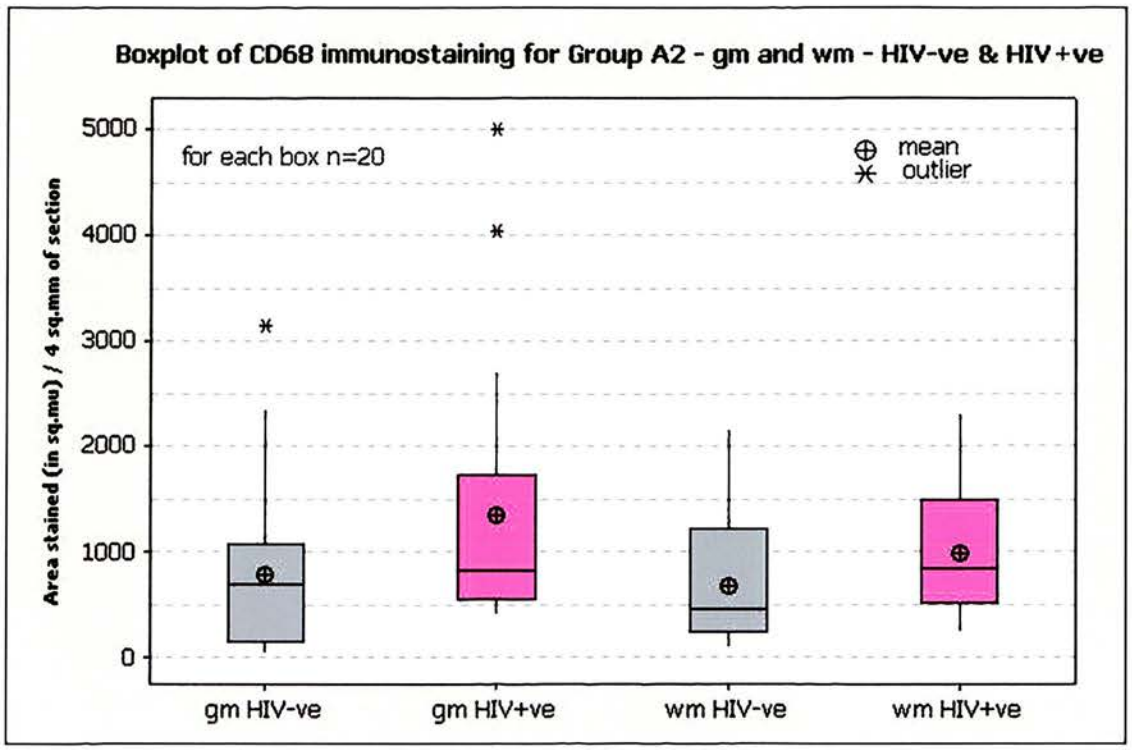
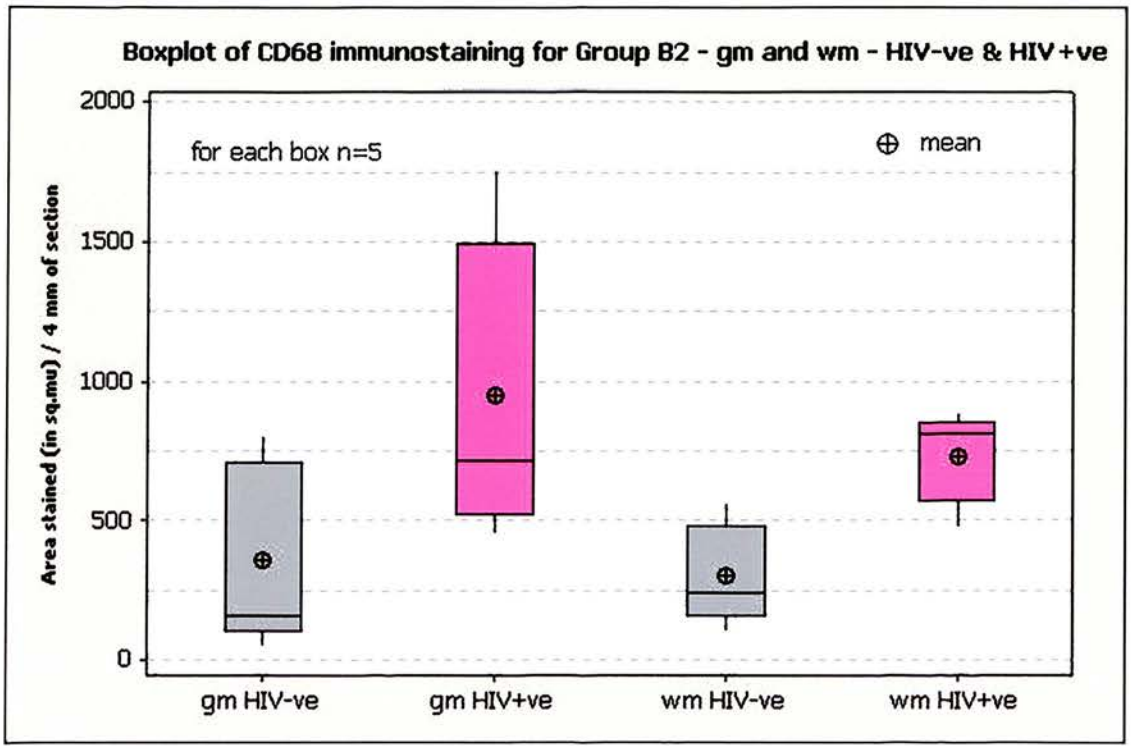
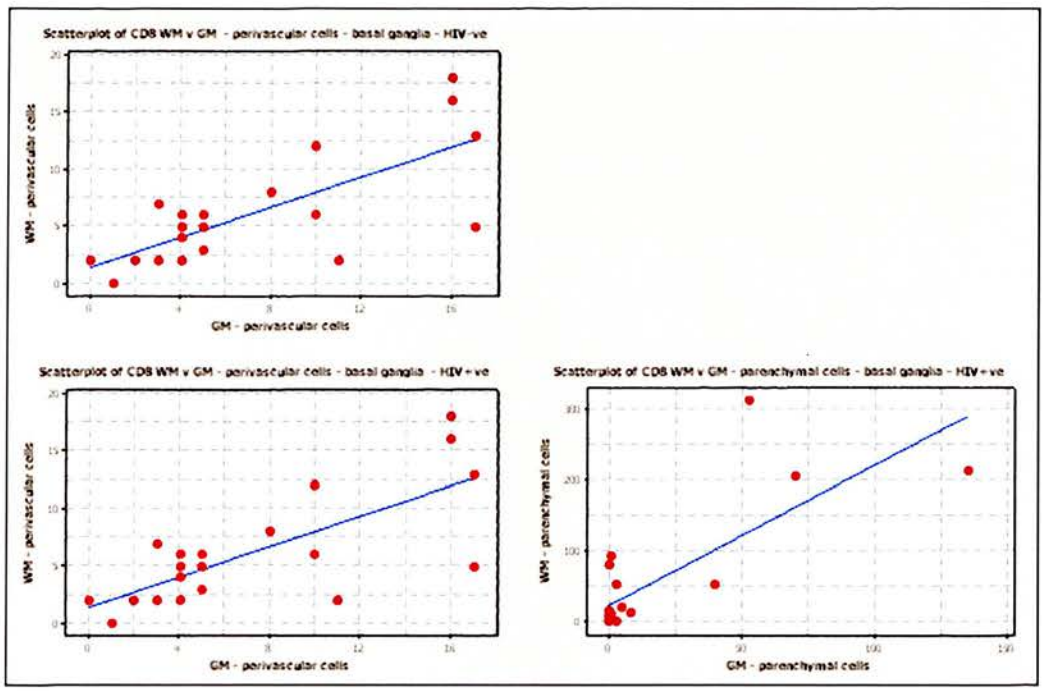


Fig. 4.3.38 HC CD68 Group B2 – boxplot of image analysis for grey matter and white matter



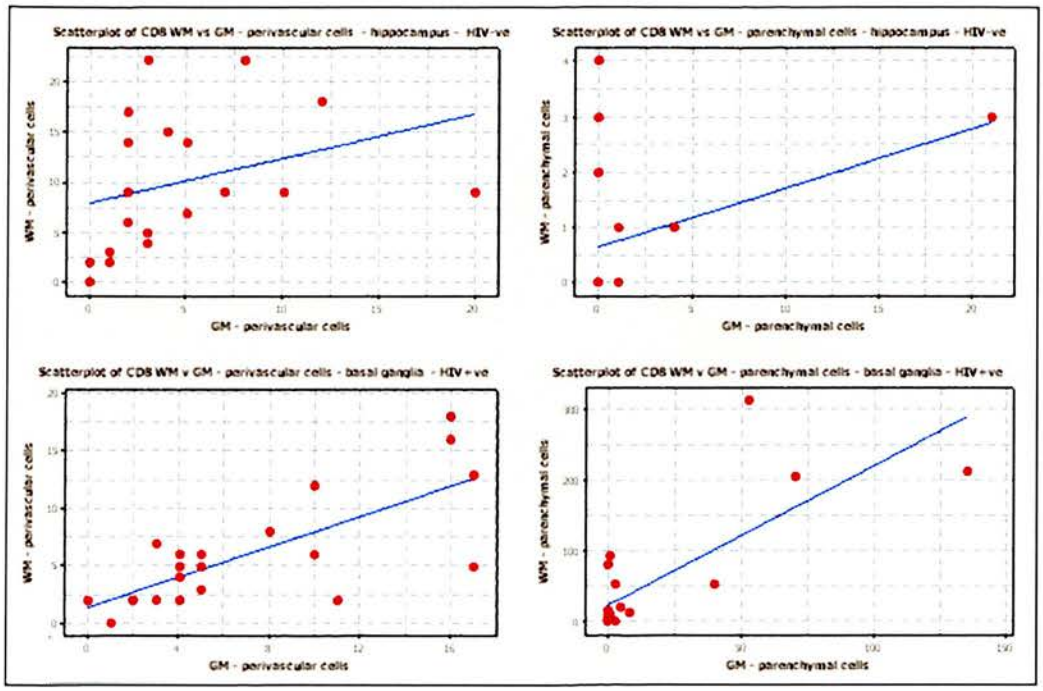
In the following scatterplots the HIV negative cases are in the upper half of the figures and the HIV positive in the lower half. In the first figure, Fig.4.3.39, there is no plot for CD8 parenchymal cells as only four values were recorded in the grey and white matter, and only two of these were matched.

Fig 4.3.39 Scatterplots of CD8 counts for basal ganglia



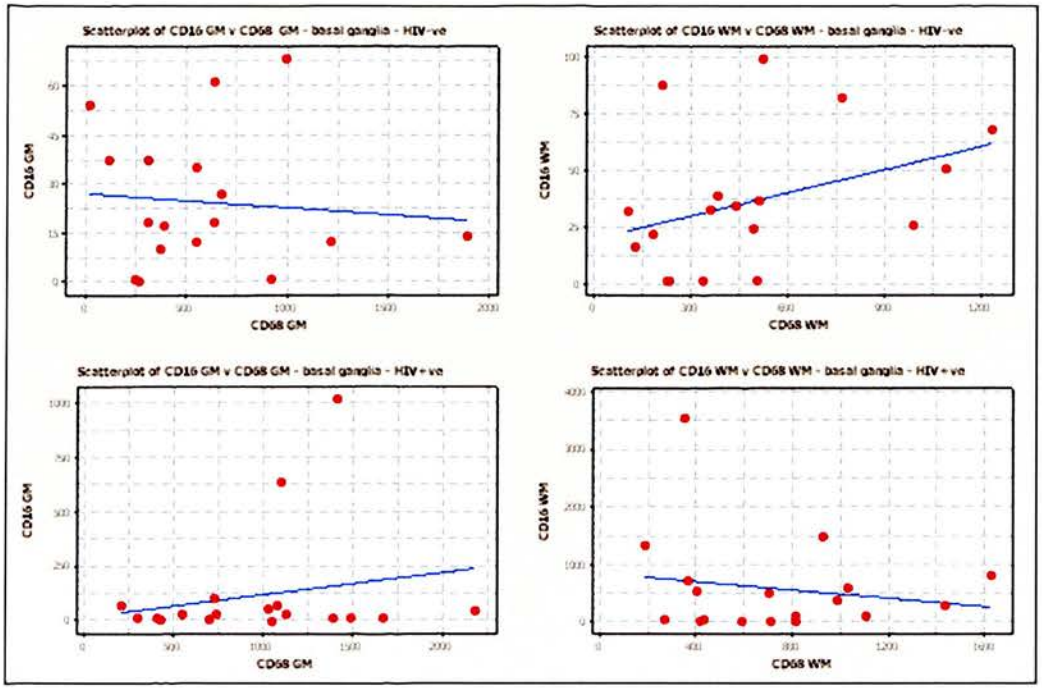
HIV negative cases (upper frame) – white matter vs grey matter for perivascular cells only. HIV positive cases (lower frames) white matter vs grey matter for perivascular (lower left) and parenchymal cells (lower right)

Fig 4.3.40 Scatterplots of CD8 counts for hippocampus



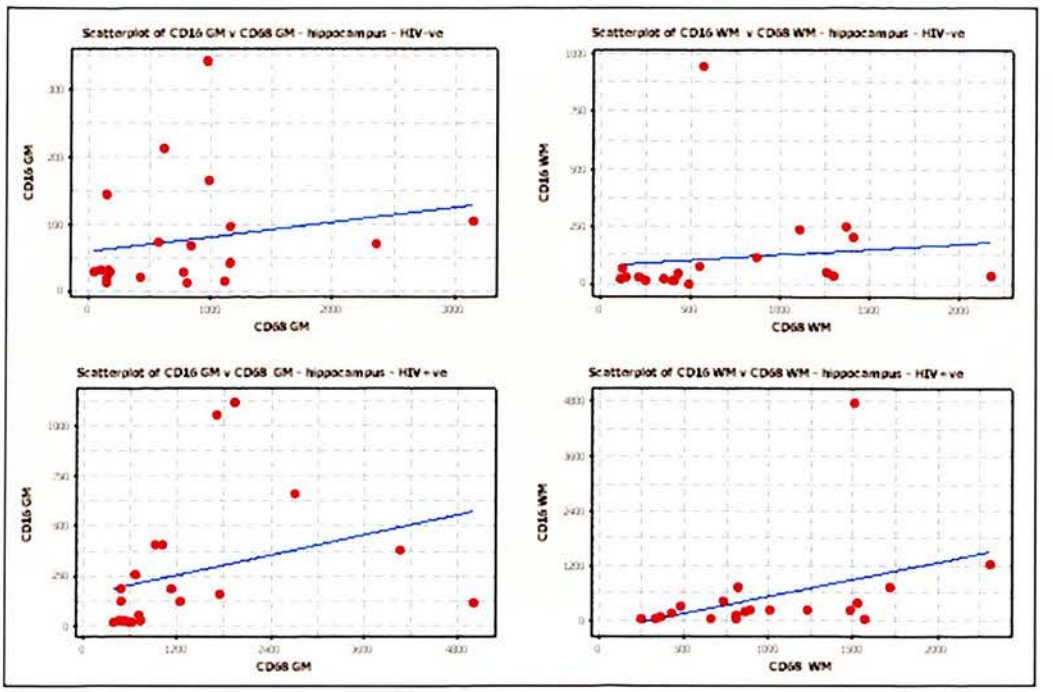
White matter vs grey matter for HIV negative cases (upper frames) and HIV positive cases (lower frames), perivascular cells (on left) and parenchymal cells on right)

Fig 4.3.41 Scatterplots of CD16 vs CD68 immunostaining for basal ganglia



Grey matter (upper left), white matter (upper right) for HIV negative cases. Grey matter (lower left), and white matter (lower right) for HIV positive cases

Fig. 4.3.42 Scatterplots for CD16 vs CD68 for hippocampus



Grey matter (upper left), white matter (upper right) for HIV negative cases. Grey matter (lower left), and white matter (lower right) for HIV positive cases

4.4 - Discussion

Introduction

Limitations of the study

When analysing the data from this study there are several caveats that must be acknowledged. Firstly the number of cases studied is relatively small and the brains of the HIV negative children are not all entirely normal. Construction of the basal ganglia and hippocampus subsets, determined by age and HIV status, necessarily shows a differing spectrum of disease between the HIV negative and positive groups. However, there appears to be a reasonable balance of pathology between the two groups and a random array, in this sense, may provide a more valid approach to comparison and analysis. Even if an attempt had been made to match conditions between the HIV negative and positive groups, for example with bacterial meningitis, there would have been difficulties. How could this be done in a meaningful way without, at the very least, knowledge of the pathogen, the presence or absence of co-existing disease, the duration of the illness and clinical findings?

Statistical analysis

The datasets for the Main Study generally did not conform to a normal distribution and were all analysed by the non-parametric method, the Mann-Whitney test (following a statistician's advice), and using Minitab statistical software. The data were displayed using boxplots for both the basal ganglia and the hippocampi. The problem of multiple analyses was discussed with a statistician who felt that the small

scale of this study and the variability of the data were such that a more sophisticated analysis was unlikely to yield more reliable results. Perhaps the best that can be said is that the results obtained serve as pointers to a likely enhancement of immune responses, particularly in the CD68 response in HIV positive subjects (in the absence, apart from the cases of HIVE, of evidence of productive infection), but that a larger survey would be required to confirm this. Nevertheless, given the degree of pathology in the HIV negative group, some of it infective/inflammatory in nature, the results for the HIV positive group are quite striking.

General Pathology Findings

The relevant general pathology findings are tabulated in Initial Study, Table 3.2.2 and this, the Main Study, Tables 4.2.2 and 4.2.3. They are, very briefly expressed, the principal causes of death. Apart from distinctive conditions, such as malaria, it seems unwise to attempt any correlation with the CNS findings. Without clinical and laboratory data how could it be said that the pneumonia, which caused the death of so many, was a "homogenous" condition

Inflammatory cells in the brain – summary of results

The CD8 results for the basal ganglia and hippocampus, Groups A1 and A2, (Table 4.3.6) revealed significantly higher values for perivascular and parenchymal cell counts in the HIV positive sets. In the smaller Groups BI and B2 the only significant results were for the parenchymal cell counts, which were greater in the white matter of both HIV positive sets. The scatterplots (Figs 4.3.39 and 4.3.40, suggest a reasonable degree of correlation between the perivascular cell counts for grey and

white matter of HIV negative and positive cases in the basal ganglia. In the hippocampus the situation is less clear cut, although values are greater in the HIV positive group. In all, the presence of outliers tended to distort the graphs. The correlation between the grey and white parenchymal cell counts for HIV positive cases was similarly difficult to interpret.

No significant difference was detected for CD20 cell counts between HIV negative and HIV positive cases, in either of the Groups for basal ganglia or hippocampus (Table 4.3.7). Similarly, there was no significant difference between the CD14 grades for HIV negative and HIV positive cases in either Group A or B, in basal ganglia or in hippocampus. Image analysis values for CD16 (Table 4.3.9) were significantly higher in the white matter of HIV positive cases for Group C basal ganglia, and in both grey and white matter of the hippocampus (GroupA2). No significant difference was detected for Group B sets in either locus. A significant increase in CD68 values was found in the basal ganglia of HIV positive cases compared with HIV negative cases, in both grey and white matter. The only other significant result was for Group B1 in the white matter of the hippocampus.

The scatterplots, Figs 4.3.41 and 4.3.42, for C16 values compared with those for CD68 values in the basal ganglia show little evidence of correlation except perhaps in respect of the grey matter in HIV positive cases. In the hippocampus the better correlation is for the white matter of both HIV negative and positive cases.

In this study there were only two HIV positive cases that could be classified definitely at the stage of AIDS. One, **c91**, was characterised by the finding of cerebral toxoplasmosis and was common to both basal ganglia and hippocampus groups. The other, **c152**, was defined by HIVE and toxoplasmosis and this case was

found only in the basal ganglia group. Without clinical details and CD4 counts it was impossible to know how many cases of probable pre-AIDS were in the subsets, but it may be inferred that at least some cases with a low grade lymphocytic infiltrate in the brain had died before the onset of AIDS. If so, the degree of CD8 lymphocyte infiltration may not be surprising but that of CD68 microglial activation is more unexpected.

Discussion of Individual Staining Results

In general the **Luxol fast blue** staining did not reveal evidence of significant white matter damage. The paler sections were usually those of children under the age of one year. There did not appear to be any relationship with HIV status or pathology findings. In view of the confounding factor of incomplete myelination, and the uncertainty as to the ages of the children and their state of nutrition, it might be unwise to place too much reliance on this data. In any case the assessment of subtle grades of pallor is a highly subjective process.

Glial fibrillary acidic protein: There did not appear to be any clear-cut relationship between the degree of activation and age, or pathological condition in HIV negative or positive Groups. In the HIV negative group, strong GFAP expression was seen in apparently normal cases and expression was not of the highest values in HIVE. In any case the results in this study are in keeping with the relative lack of white matter damage as in the LFB and βAPP grading.

β-amyloid pre-cursor protein (βAPP): The very limited positivity detected is in keeping with the results for the LFB and GFAP grading results. The overall lack of white matter reaction may be due to the early demise of these children before axonal

and myelin damage had had time to develop. Examination of further cases in the cohort might be helpful to confirm or refute the relative lack of white matter change.if the study was extended.

Discussion of Lymphocyte Studies

The behaviour of lymphocytes with respect to the CNS was studied in the rat in 1991(Hickey et al. 1991). Naïve T lymphocytes or lymphoblasts introduced into the circulation were found to enter the CNS and reach peak levels within 12 hours and leave in one to two days. The access of activated T-cells is more restricted but when entry has been achieved the tendency is for them to remain, especially if their cognate antigen is detected. Cerebral endothelial MHC class I antigen presentation is thought to play a key part in their transit (Galea et al. 2007).

The twenty **HIV negative cases** provided a spectrum of conditions in which the lymphocyte responses could be observed. In view of the background of systemic disease in this cohort it was unlikely that any more than a few cases would reflect the baseline levels for **CD8** lymphocytes in this population. The counts for case **c93** can probably be taken as basal levels. Several of the cases with no significant findings on initial examination showed elevated counts, for example, **c50** and **c67**, and it seems likely that systemic disease might account for these results. It is of interest that individual cases show discrepancies between the CD8 and CD68 results. For example, the case of possible septicaemia, **c33**, showed high values for CD68 expression but had low counts for CD8 lymphocytes, in both basal ganglia and hippocampus. The finding that some of the cases of purulent meningitis, for example **c109**, had such low counts for CD8 cells might be explained by a short time course

for an overwhelming infection (but note the low CD68 results also). The CD8 cell counts in the cases of cerebral malaria differed slightly, **c117** showing lower levels, but both malaria subjects were older children (aged 2.9 and 6 years) and may have developed a degree of immunity. These cases may suggest that different pathologies may induce primarily a lymphocytic or macrophage response.

The immune responses to malaria are complex and may take years to develop fully (Good et al. 2005). The role of CD8 lymphocytes has been investigated in experimental cerebral malaria in mice and depletion of CD8 T-cells or splenectomy have been shown to have a protective effect (Hermsen et al. 1998). One way in which CD8 cells are thought to contribute to the pathogenesis of cerebral malaria is by increased adherence to the cerebral capillary endothelium (Nitcheu et al. 2003) (Fig. 4.3.6). The lower counts in **c117** may therefore be of prognostic significance.

The role of CD8 T-cells in the CNS in **HIV positive cases**, appears on available evidence likely to be multifactorial. HIV-specific cytotoxic T-cells are present in the sera of HIV infected individuals (Walker et al. 1987) and are involved in the suppression of HIV in the acute and chronic stages of the infection (Brander and Riviere 2002) . They are also known to be infected from early in the infection (Livingstone et al. 1996) . The question of timing of viral entry into the CNS, and therefore a lymphocytic presence, is one of continuing debate (McCrossan et al. 2006). Certainly significant numbers of perivascular CD8 lymphocytes are present in the brains of HIV infected pre-symptomatic individuals (Bell et al. 1993) while an underlying low level of pro-virus is detected by limiting dilution nested PCR (McCrossan et al. 2006). The relationship is considered to be one of control by the CD8 lymphocyte population and the precise factors which lead to the loss of control

and the development of productive infection remain undefined. The root cause of progression of HIV/AIDS is generally accepted to be the increasing degree of immune suppression. In this regard, the introduction of HAART has resulted in some changes which are not well understood. One of these is the continuing inflammation in the CNS, as detected by macrophage/microglial activation in an autopsy study (Anthony et al. 2005) in post-HAART subjects (which, as in this present study, focused on the basal ganglia and hippocampus). The other is the as yet incompletely explained and lethal phenomenon of the immune reconstitution inflammatory response (IRIS) in which, in association with HAART, there is a great influx of CD4 and CD8 lymphocytes to the CNS (Miller et al. 2004) .

The twenty HIV positive cases in this study were probably infected perinatally. The two cases that had progressed to definite AIDS were aged 0.4 and 6.4 years and illustrate the age spectrum in childhood AIDS, from the many who succumb in the first two years to those who survive to mid to late childhood. The bimodal distribution of AIDS presentation in children in the USA is not considered to be so applicable to children in sub-Saharan Africa because of the high background of infectious disease.

An increased quota of perivascular and parenchymal CD8 cells was confirmed in HIV positive cases in this study. As mentioned earlier, the number of pre-AIDS subjects in this study is unknown but it is considered that some, at least, of the cases displaying low-grade lymphocytic infiltrates were at this stage of HIV infection. Indeed 4 of the 6 cases had high parenchymal counts in the basal ganglia and all had high perivascular cell counts in both grey matter and white matter. Four of these children were under the age of two years at death. It is concluded that the level of

CD8 influx reflects the balance of disease and adaptive response in the particular child at the time of death, recognising that no two human subjects are the same.

The rarity of **CD20** cells in the parenchyma of **HIV negative cases**, both for the basal ganglia and hippocampus, is in accordance with what is known for the normal adult nervous system - namely that they are present, but in very small numbers (Anthony et al. 2003). There is no reason to suppose that children should differ in this respect. The elevated counts in cases such as **c50**, with no significant findings, **c54**, a case of meningoencephalitis with infarcts, and **c60**, with no significant findings, suggest responses to both local (in the case of **c54**) and remote (**c50**, **c60**) stimuli.

Among the **HIV positive cases,** the case of measles encephalitis, **c24**, is remarkable for the absence of CD20 positive cells in either the basal ganglia or hippocampus. Furthermore, although there are elevated CD8 cell counts, these are quite modest compared with those for other cases in the CD8 HIV positive group. Measles is usually accompanied by a brisk immune response in the blood, involving both CD8 T cells (cytotoxic T lymphocytes) and CD20 cells (B lymphocytes). Also, in encephalitis (non-HIV), there may be significant numbers of CD20 cells in both the parenchymal and perivascular compartments (Anthony et al. 2004). Perhaps there was a reason other than HIV for this B cell absence, such as severe malnutrition, which is known to impair the immune response (Whittle 1996)

A similar lack of CD20 response, striking in the case of the basal ganglia and more limited in the case of the hippocampus, applied in the AIDS case, **c110**. But in this case there were high parenchymal and perivascular CD8 counts in both the basal ganglia and hippocampus. In the other AIDS case, **c152**, a fairly high count was seen

in the basal ganglia white matter, with a negligible response in the grey. These results should be seen in the context of what is known about the B-cell numbers in the brain in AIDS and HIVE, namely that they tend to fall to below normal levels in the progression towards AIDS (Anthony et al. 2004).

Several of the cases showing low-grade lymphocytic infiltrate had CD20 counts above the means for the group. For instance, in the basal ganglia of **c55** and **c62** and in the hippocampus of **c62**, **c70** and **c91**, the counts are several times greater than the means. Increases in numbers of CD20 lymphocytes in the brain of HIV positive adults may herald the pre-AIDS phase (Anthony et al. 2003). Perhaps this increase in CD20 counts in the brain is a surrogate marker for the imminent onset of AIDS in some of these cases, especially where there are also high values for CD68 expression, for example **c55** and **c91**.

Discussion of the Macrophage and Microglial Markers

The grades for **CD14** immunostaining in the **HIV negative cases** tended to be higher in the cases where there was evidence or suspicion of intravascular or perivascular infection. In particular, these included the possible septicaemia cases, and the cases of meningitis and of malaria. This is in keeping with the role of CD14 cells as gate-keepers of the CNS. In the HIV positive cases higher values were seen in both AIDS cases and some of those with low- grade lymphocytic infiltrates. As in the HIV **negative group, the case of purulent meningitis has higher grades.**

The results for **CD16** quantitation appeared to parallel those obtained for CD68 and the results are considered together. In the **HIV negative cases,** the difference in **CD 68** values between basal ganglia and hippocampi may simply reflect a natural

variation in disease expression from one brain region to another, as was found in another study which identified a similar variation in inflammatory response in HIV infection in the basal ganglia and hippocampus (Anthony et al. 2005).

The molecular mechanisms that determine inflammatory cell access to the CNS appear to vary with respect to both stimulus and site – whether this be cerebrum or spinal cord, grey matter or white, and this subject was examined in a review in 2005 (Engelhardt and Ransohoff 2005). These considerations may apply to other cases such as cerebral malaria, as in **c104**. The values for CD68 expression in this case were much higher in the hippocampus than in the basal ganglia. Another factor is that severity of malarial disease in West African children is known to be strongly correlated with serum levels of TNFα (Kwiatkowski et al. 1990) and thus with potential damage to the blood brain barrier. The highest values recorded in the HIV negative group for both sets were those for the possible septicaemia cases **c23** and **c77**. As there was no corresponding significant rise in the lymphocyte counts these results can be interpreted as a rapid response to an acute systemic infection. However the results for the cases of purulent meningitis introduce a paradox. These results were in the low or medium range of values and yet the infection was clearly both in very close proximity and intense (compare Figs. 4.3.12. and 4.3.14). The explanation for this is uncertain without knowledge of clinical or laboratory findings. In part it may be explained by differing pathogens or time courses, an impaired state of immunity due to causes other than HIV, and perhaps even attributable to changes in the integrity of the blood brain barrier.

In the **HIV positive cases**, CD68 staining was significantly accentuated, compared with the negative cases. Of the 2 definite AIDS cases, **c110**, with toxoplasmosis and

aged 0.4 years, showed generally high levels of staining while the other, **c152**, with HIVE showed only moderate CD68 staining (despite high values for CD14 and CD16), These two cases were not really comparable. Firstly there was the age difference - the infant could be seen to be in a rapid progressors group, possibly signifying intra-uterine infection. The supposition was that the toxoplasmosis was congenital. In contrast, the older child had HIVE, and the relatively modest CD68 staining levels were perhaps surprising, but the prominent CD14 and CD16 results were in keeping with what is known of responses in HIVE.

High levels of CD68 expression were seen in about half of the cases showing a low-grade lymphocytic infiltrate and in most of these there was also increased expression of CD16. Four of the six cases in this category were under the age of 2 years at death and it is likely that some if not most of these cases in this category were cases of pre-AIDS

Some of the cases with no significant findings on initial examination showed high levels of activation and presumably the causes for these were systemic, possibly infection in the pulmonary or gastro-intestinal systems.

In this discussion of the macrophage/microglial contribution to the inflammatory responses of the CNS, reference is briefly made to the part probably played by the choroid plexus. A study of the choroid plexus in HIV infection showed that of 25 AIDS cases, 70% contained T lymphocytes and 50% contained monocytes (Falangola et al. 1995). Almost half contained HIV positive monocytes and it was suggested that this finding supported the hypothesis that HIVE might develop from such a source. In this study the choroid plexus was frequently seen in hippocampus

sections and both lymphocytes and macrophages were detected in the plexus, by CD8 and CD68 immunostains respectively (see Figs. 4.3.17 and 4.3.18)

With regard to the results obtained from the B Groups, (n=10, 5 HIV negative and 5 HIV positive), in which it was hoped that the effects of other confounding pathology would not affect results due to HIV alone, it is noted that, for the most part, the results fell short of significance apart from CD8 and CD68 comparisons, reflecting the large differences for these parameters in the larger groups. This was probably due to the small sizes of Groups B1 and B2 but unfortunately few of the original cohort were in the category of having no significant neuropathology on routine examination.

General Discussion of Results

The results for the markers of macrophages and lymphocytes confirm that there is significant entry of inflammatory cells into the CNS and activation of the microglial population, in the absence of detectable intrinsic infection by HIV. Furthermore there is influx and activation in HIV negative cases, even without manifest disease of any kind in the CNS and, paradoxically, there is little or no response in the presence of intense local surrounding infection, as in the HIV negative cases with purulent meningitis. Despite these potentially confounding factors, there is significant microglial upregulation in the HIV positive group.

In the past few years there has been sustained interest (Perry 2007; Perry et al. 2007), in the question of microglial activation in relation to systemic disease and the cognitive correlates, especially in respect of neurodegenerative disorders, such as Alzheimer's disease. It has been shown that even minor infective illness elsewhere

in the body can alter CNS function and lead to behavioural change. Of course such an alteration could be due to neuro-synaptic interference (Epstein and Gendelman 1993) and the evidence suggests that the microglial population is involved and may indeed be the prime mover through cytokine release. The main question seems to be one of access to the brain parenchyma in several contexts – why do only a proportion of AIDS cases suffer from CNS involvement? Why, if there are significant responses to minor systemic illness, is there so little involvement when there is intense local inflammation, as in meningitis - even allowing for a short time course in such illnesses? The answer must lie, at least in part, in the blood brain barrier. Just as there is regional specialization in capillary endothelium (Engelhardt and Ransohoff 2005), might there not also be genetic influences at work conferring resistance or otherwise to immune cell access ?

The intense microglial activation detected by CD68 staining in the cases of possible septicaemia serves to focus attention on the dynamics of immune cell responses, which occur in most diseases affecting the CNS (Kreutzberg 1996). However the role of immune activation in the pathogenesis of many CNS conditions remains unclear (Weiner and Selkoe 2002). It is a widely held opinion that the effects of microglial activation are malign in HIV infection, and it is thought the damage in HIV encephalopathy is due to cytokine secretion by activated macrophages (Yoshioka et al. 1995). The principal molecule involved is TNF-α.

The role of macrophage activation in CNS disease has been explored in mice by a technique known as microglial paralysis, which involves genetic manipulation such that microglia are developed that are incapable of producing proinflammatory

cytokines. In such mice, the response to facial nerve section was blocked and the response in experimental allergic encephalitis suppressed (Heppner et al. 2005).

Since the introduction of HAART and the great improvement in mortality figures, there have been fewer large studies on the neuropathology of HIV infection in children or adults (Vallat-Decouvelaere et al. 2003), and not many address the problem of microglial activation. A recent post mortem study on adult brains examined microglial activation, detected by CD68 immunostaining, in the setting of drug abuse. The results for the normal controls were by image analysis (pixels per unit area) in the 200-1000 range (Arango et al. 2004). The highest levels, for HIVE subjects who were drug abusers, were in the 2,000 to 4,500 range, and for HIV non-drug users the range was similar but with lower means.

In the present study macrophages with a rectangular nuclear profile were seen in both HIV negative and positive cases that showed a high level of activation (Fig.4.3.10). Some of these were positively stained by the macrophage/microglial markers and others were not. There was, and still is, debate concerning the naming and the origins of these cells (Dickson et al. 1991). They are variously described as "activated" or "amoeboid", and they may be derived from both microglia and perivascular macrophages. Their appearance, especially in the white matter, is suggestive of determined movement, and tissue culture studies have revealed their dynamic properties (Esiri 1996).The very early responses of microglia to changes in the CNS have been revealed by in-vivo experiments involving two-photon laser scanning microscopy (Nimmerjahn et al. 2005). In this technique cortical microglia in mice were visualized directly, both in their resting and early activated state. At rest, microglial processes were seen to be in a constant state of expansion and

retraction of the protrusions on these processes. After a micro-injury induced by a two-photon laser, the processes of neighbouring microglia were observed to enwrap the zone of damage whilst retracting the processes that extended in the opposite direction. The authors emphasised that the function of microglia is primarily one of neuroprotection and that the effects of microglial activation depend on the nature and intensity of the stimulus.

In general, in viral infections of the CNS, macrophages, CD8 T-cells and natural killer cells tend to move into the parenchyma, whereas B-cells and CD4 T-cells usually remain in a perivascular position (Esiri 1997). In the HIV negative cases in this study there was little parenchymal distribution of CD8 T-cells, but in the HIV positive group, as noted above, both AIDS cases showed appreciable numbers (Tables 4.3.4 and 4.3.5). However large numbers were also seen both in grey and white matter of the cases with low grade lymphocytic infiltrate, and this is rather different from the findings in another study in which CD8 T-cells were rare or absent in non-HIVE cases (Petito et al. 2006). If, as these authors propose, CD8 T-cells could be an important biomarker for productive brain infection by HIV-1, it might be inferred that some, at least, of the cases in the low grade lymphocytic infiltrate category are in the pre-AIDS phase. Another study which considered grading of CD8 cells in the parenchyma showed a significant presence of CD8 lymphocytes in the hippocampus in both pre-symptomatic and HIVE cases, but slightly lower in AIDS (Anthony et al. 2005).

In conclusion brief mention is made of two other conditions which feature in the main study, cerebral malaria and measles encephalitis.

Cerebral malaria is a serious cause of death world-wide, causing up to 2 million deaths annually (Medana et al. 2001). The pathophysiology is not well understood, particularly as the CNS is not actually invaded by the parasite, which remains intra-vascular. In the past, hypoxia due to obstruction in the microcirculation was thought to be the major factor, but now the focus is on damage to the blood brain barrier (BBB) and the secondary sequelae associated with protein seepage and increased inflammatory cell access (Szklarczyk et al. 2007). The two cases included in this study show significant immune activation only in one. The difference may simply be due to a shorter time course or co-existing disease.

Measles encephalitis is seen infrequently in Europe and the USA, now that effective immunisation programmes are in place. In the developing world, measles is still a major cause of morbidity and mortality, but in Africa the estimated mortality has fallen, due largely to the introduction of community based immunisation programmes, that have contributed 72% of the total global disease reduction (Wolfson et al. 2007) The high level of inflammation shown in the single case in this study underlines the severity of the response to viral infections other than HIV infection. The results of a small study, also based in Abidjan, suggested that HIV positive children might be more susceptible to CNS involvement by measles virus (McQuaid et al. 1998).

Conclusions

1 Very **limited evidence of white matter damage** was detected in this study by the methods used, namely Luxol fast blue routine staining and immunostaining using GFAP and βAPP.

2. Compared with HIV negative children, positive children had significantly higher levels of **microglial activation** in the white matter of both basal ganglia and hippocampus, as detected by CD16 immunostaining.

3. Compared with HIV negative children, positive children had significantly higher levels of **macrophage/microglial activation** in the grey and white matter of the basal ganglia, and in a small subset of the hippocampus, by CD68 immunostaining.

4. Compared with HIV negative children, positive children had significantly higher levels of **lymphocyte infiltration** in both grey and white matter of the basal ganglia and hippocampus, and in both perivascular and parenchymal loci.

Chapter 5: The APOE Study

5.1- Introduction

History: major milestones in the understanding of APOE and its relationship to human disease

Darwin's publication in 1859 of "The Origin of Species" (this year marking the 250th anniversary of publication) resulted in a completely new understanding of all living things. The concept of "survival of the fittest" described how the members of a population who are better adapted to their environment tend to flourish and pass on their advantageous traits. The mechanism of this "passing on" was to be explained in a simple and lucid way by Mendel in 1856 as a result of his experiments on the breeding characteristics of the edible pea. In 1953 Watson and Crick published their proposal of the double helix structure for DNA, which now underpins our present understanding of molecular biology (Watson and Crick 1953).

A further fundamental discovery, which has transformed the field of molecular genetics by facilitating the amplification of DNA from a single molecule to a hundred billion copies of itself in a few hours, was the discovery of what is now known as the polymerase chain reaction (PCR) (Mullis et al. 1986). In essence this is the catalysed incubation of oligonucleotide fragments with the enzyme DNA polymerase.

Since these studies the field of genotyping has expanded enormously, and molecular biology now allows a very sophisticated understanding of the genetic determinants of disease. In the field of neuroscience many studies have attempted to

link genes with disease. In Alzheimer's disease (AD) one of the strongest genetic links has been shown to be the apolipoprotein (APOE) gene, found on chromosome 19 (Burke and Roses 1991). This gene encodes a protein of the same name. Here the convention of APOE is adopted for the gene and ApoE for the protein.

Biochemistry and Genetics

Lipoproteins are macromolecules involved in the transport of lipids, such as cholesterol and free fatty acids, in the bloodstream. The name apolipoprotein is derived from the use of the Greek prefix, Apo – off, from, away - and refers to the protein moieties on the surface of the macromolecule. On this basis several different apolipoproteins are recognized, of which ApoE is of particular interest because of its involvement in CNS homeostasis and disease. ApoE is a complex molecule with a molecular weight of 34 KD and is composed of 299 amino acids (Rall et al. 1982). The APOE gene exists in three allelic forms and codes for corresponding protein isoforms, ApoE 2, 3 and 4, which differ in their arginine and cysteine content (Zannis et al. 1981). The alleles are known as APOE ε2, APOE ε3 and APOE ε4. This allelic variation allows six genotypes – three homozygous (ε2/ε2, ε3/ε3 and ε4/ε4), and three heterozygous (ε2/ε3, ε2/ε4 and ε3/ε4). The distribution of the alleles has been the subject of many studies. For example a study in Scotland found the following distribution in adults aged 45-60 years – ε2 in 8%, ε3 in 77% and ε4 in 15% (Cumming and Robertson 1984).

The ε3 allele is the commonest variant and occurs at a frequency of 60-80% in most Western populations. The frequency of APOE ε4 is fairly constant at 10 -12% throughout southern and western Europe but reaches 30% in Scandinavia (Mahley

and Rall 2000). In Africa the overall distribution APOE alleles is different from Caucasian populations with APOE ε2 found in 11.6%, APOE ε3 in 70.6% and APOE ε4 in 17.8%. The frequency of APOE ε4 varies from 9% to 41% across Africa (Zekraoui, l – 1997) and in Nigeria, West Africa, a frequency of 22-29% was recorded for APOE ε4.

It is now well established that there is a strong association between the development of AD and possession of one or more APOE ε4 alleles (Corder et al. 1993). In contrast, no such association was found between APOE ε4 and AD in elderly Nigerians (Osuntokun et al. 1995).

The issue as to which is the ancestral allele is pertinent to the West African population on which this study is based. In the early years it was considered likely that it was the APOE ε3 allele because of its frequency (Mahley 1988). However, since Africa was the site of origin for mankind and shows a higher frequency of APOE ε4, there are reasons to conclude that this is the more likely candidate. Supporting evidence comes from gene sequencing in non-human primates (Hanlon and Rubinsztein 1995), and from other African studies (Zekraoui et al. 1997). It is possible that possession of APOE ε4 alleles may have conferred a selective advantage in the form of increased capacity to survive in an environment so rich in pathogens, particularly the protozoal group (Sinnis et al. 1996).

Physiology

ApoE protein is expressed in a variety of cells in different organs including the liver, kidneys, spleen and brain. The protein is involved in cholesterol metabolism, cell repair and especially in immune activation. The integral role of APOE in lipid

metabolism underlies an association with the development of atheroma and the pathogenesis of ischaemic heart disease. The level of ApoE protein in the blood is 30-70 μgm/ml; most of this is produced in the liver. High levels are also expressed in the brain (Mahley and Rall 1999). A study in which tissues were screened for ApoE messenger RNA (mRNA) found high concentrations in all areas of the brain, as in the liver, in keeping with active protein production (Elshourbagy et al. 1985). A further study showed that ApoE protein was associated exclusively with astrocytes (Boyles et al. 1985), either within these cells or on astrocyte processes ending at the glia limitans or on blood vessels. This finding has been confirmed (Fujita et al. 1999).

In AD the hallmark at microscopical level is the presence, in the cerebral cortex, of senile plaques and neurofibrillary tangles. Microglia are frequently seen in association with these plaques and a causative role has been suspected (Mackenzie et al. 1995). The main constituent of the plaques is Amyloid-β, a 39-43 amino-acid polypeptide. It is derived from cleavage of beta-amyloid pre-cursor protein (βAPP). Microglial activation seems to occur especially near plaques expressing a high level of βAPP (Griffin et al. 1995).

The relationship between amyloid-β and ApoE protein has been the subject of much discussion. High affinity binding occurs with ApoE 4 protein, resulting in the formation of insoluble high-molecular weight compounds and, in vitro, dense amyloid monofibrils (Sanan et al. 1994). ApoE 3 protein binding and molecular elaboration occurs to a lesser degree (Wisniewski et al. 1994). Amyloid protein deposition is known to occur in the brain after head injury (Roberts et al. 1991) and this is more prevalent in those with APOE ε4 allele(s) (Nicoll et al. 1996).

The relationship between the APOE gene and AD posed the question as to whether or not there would prove to be a relationship between the APOE gene and HIV-associated dementia. A retrospective autopsy study investigating the APOE genotype of 132 HIV positive individuals (Dunlop et al. 1997) showed no association between HIV encephalitis (HIVE) and different APOE alleles. The following year the results of a clinical study on 42 HIV-infected subjects, followed for up to five years, were published. This study showed that there was a twofold excess of patients with the APOE ε4 among those who developed dementia (Corder et al. 1998). It was concluded that a partly genetically determined pathway might be common to AD and HIV associated dementia (HAD) or alternatively, a gene–virus interaction might be operating to accelerate AD pathogenesis.

Lipoproteins were shown to have a role in immune regulation in 1976, when a low density lipoprotein was shown to be capable of inhibiting lymphocyte proliferation (Curtiss and Edgington 1976). ApoE protein was then shown to be the most important lipoprotein in this respect (Hui et al. 1980).

Since 1997 there has been increasing interest in the relationship between APOE, ApoE protein and β amyloid derivatives on the one hand, and the innate immune system of the CNS on the other. In tissue culture experiments using neuronal-glial preparations, the secretion of tissue necrosis factor alpha (TNFα) was shown to be related in a dose-dependent way to the presence or absence of ApoE protein (Laskowitz et al. 1997). In another study, soluble APP alpha (sAPP-α), a secreted derivative of βAPP, was used to investigate the inflammatory responses of microglia (Barger and Harmon 1997). Pre-treatment of microglial cultures with sAPP-α was found to upregulate activation markers and to increase production of neurotoxins.

This response was blocked by prior application of ApoE 3 but not by ApoE 4. The differential secretion of nitric oxide (NO), a potent neurotoxin produced by macrophages and microglia, was explored in relation to the different ApoE isoforms in 2002. The findings were that more NO was produced by macrophages from humans carrying the APOE ε4 allele were found to produce more NO than those from APOE ε3 individuals (Colton et al. 2002).

Turning to in vivo studies, it appears that microglia tend to be associated with dense amyloid plaques rather than diffuse plaques, suggesting that microglial activation occurs later rather than earlier in AD (Vogelgesang et al. 2002). In 2003 a report was published on the relationship between astrogliosis and APOE in transgenic mice, and the response to injected lipopolysaccharide (LPS). Enhanced astrogliosis was shown in both the control and APOE ε3 transgenic mice, but no effect was noted in APOE deficient and APOE ε4 transgenic mice (Ophir et al. 2003). Further studies using transgenic mice have confirmed the relationship between the different APOE genotypes and the degree of microglial activation, and that an apparent inhibitory effect associated with APOE ε3 was gene dose dependent (Vitek et al. 2007).

The dynamics of the influence of the APOE genotypes on innate immune responses in the CNS are still a matter of debate. In 2000 the view was expressed that APOE ε2 and APOE ε3 were active in repair processes and neuroprotection, and that APOE ε4 was relatively inactive (Mahley and Rall 2000). Since then a modified view, that APOE ε4 is associated with, and possibly a promoter of, neuroinflammation has seemed as likely a proposition (Vitek 2007). To our knowledge, there has been no

previous investigation of a possible association between HIV associated microglial activation and APOE genotype.

Accordingly the **hypothesis** for this part of the study was that possession of one or more APOE ε4 alleles accentuates HIV associated microglial activation.

The **aims** of this study were:

1. To extract DNA and determine the APOE allelic distribution, using PCR and subsequent restriction enzyme digestion applied to the 40 cases from the main study, and subsequently to 20 extra cases.
2. To relate the degree of inflammatory change in each case, as detected by CD68 and CD16 immunohistochemistry, to the different APOE genotypes.

5.2 - Material and Methods

First, the forty cases used in the Main Study (basal ganglia subset) (see Chapter 4) were subjected to DNA extraction, and subsequent PCR if the first step was successful, as described in Chapter 2. Of these 40 cases, 20 were HIV positive and 20 were negative. Subsequently, a further 10 HIV positive and 10 negative cases were selected within the age range of the Main Study set. Thus, genotyping was attempted on 60 cases in total.

In addition, immunohistochemistry for CD68 and CD16 was undertaken on paraffin sections of the basal ganglia in the selected cases, as described in Chapter 2.

Quantitation of microglial activation in these sections was undertaken as described in Chapter 4.

The data for this study was analysed using Minitab soft ware. The chi-square test was used in the analysis of the APOE allele frequencies, and the Mann-Whitney test for the image analysis data.

5.3 - Results

Of the 40 cases derived from the Main Study, 33 proved amenable to downstream PCR analysis on first extraction (Figs 5.3.1 & 5.3.2). The remaining 7 cases were re-extracted and 4 of these proved amenable to DNA amplification by PCR. Of these 37 cases in which APOE genotypes were determined, 19 were HIV negative and 18 HIV positive. A further set of twenty cases, 10 HIV negative and 10 HIV positive, was examined and of these successful genotyping was achieved for 4 in the HIV negative group and 6 in the HIV positive group. In all, APOE genotypes were ascertained in 23 HIV negative and 24 HIV positive cases (Table 5.3.1).

Fig. 5.3.1 shows an example of PCR amplification of the APOE gene, revealing a product of 220bp. Figure 5.3.2 shows subsequent restriction enzyme digest of the PCR products using Hha1 to reveal the APOE genotype. The size of cleaved products in the metaphor gel is used to determine the genotype. The APOE ε2 allele produces two main fragments of 91bp and 81bp length. Digestion of the ε3 allele results in fragments of length 91bp, 48bp and 33bp, while ε4 produces fragments of

72bp, 48bp, 34bp and 19bp. There are also two smaller fragments common to all alleles: these are 18bp and 16bp in length.

Fig. 5.3.1 - APOE PCR: Agarose gel showing initial extraction of gene product

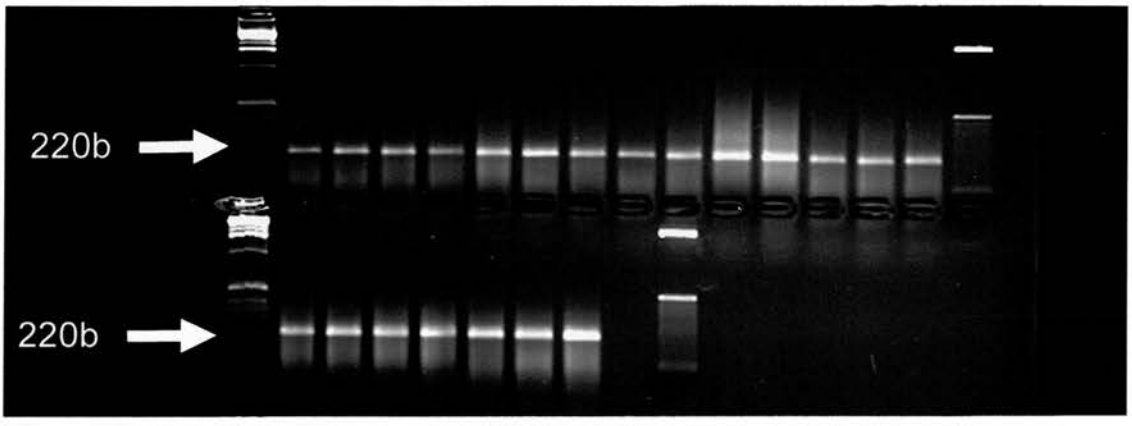
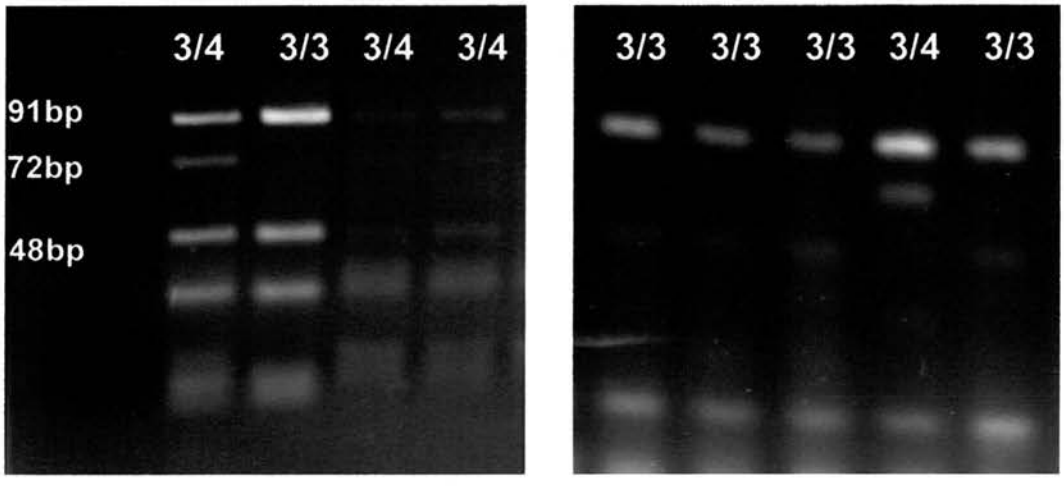


Fig. 5.3.2- Restriction enzyme digest of APOE PCR products



Key: Lane 1 and 2 control cases.

Table 5.3.1 shows the frequency of APOE ε2, ε3 and ε4 alleles in the study cohort compared with data taken from the Scottish population.

Table 5.3.1 shows the frequency of APOE ε2, ε3 and ε4 alleles in the study cohort compared with data taken from the Scottish population.

Table 5.3.1 - Frequency of APOE alleles in the African cohort compared with the Scottish population

Group	ε2	ε3	ε4
HIV negative African Children n=23	0%	59%	41%
HIV positive African children n=24	2.%	75%	23%
Scottish population* n=400	8%	77%	15%
* Data taken from Cumming & Robertson 1984			

A comparison of the HIV negative African cases with the Scottish data shows a significant difference in the allele frequencies (X^2=22.45, p=<0.001). In particular the ε4 allele was more than twice as common in HIV negative African children as in the Scottish population. In addition, there was a significant difference between allele frequencies in the two African groups, HIV positive and negative (X^2=8.97, p=0.011).

Technically satisfactory immunohistochemical staining for the markers of microglial activation, CD68 and CD16, was achieved in most but not all the

genotyped cases, including 21 of 23 HIV negative and 23 of 24 HIV positive cases analysed for **CD68** expression, and 18 of 23 HIV negative and 21 of 24 HIV positive cases for **CD16** expression.

Table 5.3.2 shows the case by case data for APOE genotype and microglial/macrophage quantitation in the selected series as well as the CNS and general pathology findings. The cases successfully genotyped from the 20 extra cases are marked in blue.

The APOE genotypes in the two HIV negative cases in which immunohistochemistry was not successful were APOE ε3/ε4 and APOE ε4/ε4, and in the HIV positive case, APOE ε3/ε3.

Table 5.3.2 APOE genotyping with CD16 and CD68 image analysis results

ApoE genotyping				2/3	3/3	3/4	4/4	CD16 BG		CD68 BG			
Case	**Age***	**Sex**	**HIV**					gm	wm	gm	wm	CNS pathology findings	General pathology 1
HIV negative cases													
c2	2.6	M	-		3/3			19	45	568	478	encephalitis	pneumonia
c21	0.4	F	-		3/3			n/a	n/a	650	437	no significant findings	CMV hepatitis oesophagitis
c27	2.5	F	-		3/3			617	483	148	119	meningitis	no significant findings
c46	1.2	M	-		3/3			39	15	256	262	no significant findings	pneumonia colitis
c50	1.6	F	-		3/3			n/a	n/a	364	464	no significant findings	pneumonia
c52	0.5	M	-		3/3			23	17	87	141	no significant findings	pneumonia pyelonephritis
c54	0.4	M	-		3/3			27	82	310	763	meningo-encephalitis & infarcts	no significant findings
c89	0.5	F	-		3/3			17	24	390	494	meningo-encephalitis	pulmonary abscess
c117	2.9	M	-		3/3			10	22	370	185	cerebral malaria	malaria
c33	1.6	F	-			3/4		12	51	1,211	1,088	possible septicaemia	pulmonary infarction
c42	5.0	F	-			3/4		1	33	923	363	no significant findings	pneumonia
c23	1.5	M	-			3/4		38	38	83	66	no significant findings	pulmonary CMV
c57	5.0	F	-			3/4		18	1	313	233	no significant findings	lymphoma, B-cell
c59	1.6	M	-			3/4		n/a	n/a	168	64	meningitis	malnutrition
c60	1.5	F	-			3/4		18	34	634	440	no significant findings	malaria pulmonary infarction
c63	2.5	F	-			3/4		27	29	76	130	no significant findings	colitis pneumonia
c76	1.6	M	-			3/4		12	39	548	383	no significant findings	colitis
c77	0.3	F	-			3/4		14	68	1,887	1,231	possible septicaemia	sickle cell crisis
c74	1.5	F	-				4/4	66	99	988	523	no significant findings	pneumonia
c67	4.0	F	-				4/4	61	26	640	989	no significant findings	pneumonia
c109	0.5	F	-				4/4	37	32	118	105	purulent meningitis	pneumonia
Total = 21 cases					n=9	n=9	n=3						
HIV positive cases													
c9	5.1	F	+	2/3				50	116	1,027	1,100	medullitis	disseminated herpes
c3	0.3	M	+		3/3			9	10	204	389	no significant findings	pneumocystis pneumonia CMV
c11	0.5	M	+		3/3			31	37	530	61	meningitis	no significant findings
c13	2.5	M	+		3/3			127	762	110	781	meningitis	pneumonia pericarditis
c14	2.2	M	+		3/3			205	620	295	131	meningitis & lymphocytic infiltrate	pneumonia
c28	0.3	M	+		3/3			255	328	233	115	CMV - wm damage	pneumocystis pneumonia CMV
c55	1.7	M	+		3/3			637	25	1,098	712	low grade lymphocytic infiltrate	pneumonia strongyloidosis
c51	1.8	F	+		3/3			n/a	n/a	107	121	low grade lymphocytic infiltrate	fatty liver
c121	1.6	M	+		3/3			1	16	431	420	cerebral oedema	pneumonia
c125	1.5	M	+		3/3			10	491	1,490	698	no significant findings	pneumonia
c126	1.5	F	+		3/3			1	16	298	269	no significant findings	TB - miliary
c128	1.8	F	+		3/3			4	100	725	187	low grade lymphocytic infiltrate	pneumonia
c129	0.4	F	+		3/3			31	46	550	433	low grade lymphocytic infiltrate	pneumonia
c131	0.4	M	+		3/3			70	531	206	407	no significant findings	CMV enteritis
c140	4.0	M	+		3/3			15	706	412	364	no significant findings	pneumonia
c8	3.5	F	+			3/4		14	598	1,394	1,030	subtle widespread wm damage	pyothorax (post measles)
c16	5.3	F	+			3/4		15	23	224	248	no significant findings	pneumonia CMV enteritis
c56	0.4	F	+			3/4		484	450	622	500	possible CMV infection	pneumonia pulmonary CMV
c62	4.0	F	+			3/4		1	14	698	592	low grade lymphocytic infiltrate	pneumonia
c122	3.3	F	+			3/4		10	812	1,672	1,628	low grade lymphocytic infiltrate	pneumonia
c24	5.0	M	+				4/4	41	300	2,180	1,435	measles encephalitis	pneumonia CMV
c82	0.2	F	+				4/4	0	12	1,050	811	cerebellar infarct/micro.hges	pneumonia
c91	1.6	M	+				4/4	n/a	n/a	1,592	1,193	low grade lymphocytic infiltrate	pneumonia strongyloidosis
Total = 23 cases				n=1	n=14	n=5	n=3						
Totals = 44 cases				**n=1**	**n=23**	**n=14**	**n=6**					n/a = not available for technical reasons	

Table 5.3.3 shows data for mean and median values of CD68 and CD16 quantitation, stratified against APOE genotype, for HIV **negative** African children. Only 3 genotypes were observed in the 21 cases studied, ε3/ε3, ε3/ε4 and ε4/ε4. For CD68 minimal differences in microglial quantitation were observed in the white matter in different APOE genotypes. However greater divergence was noted in the grey matter where the median value for subjects with an ε3/ε3 genotype was 364 compared to 548 for those with a ε3/ε4 genotype. Only minimal differences in CD16 expression were observed between different genotypes. The ε4/ε4 genotype was excluded from statistical analysis as there were insufficient numbers to allow satisfactory comparison. None of the comparisons in these HIV negative children reached significance.

Table 5.3.3 - Correlations between APOE genotype and neuroinflammation in the basal ganglia of HIV negative African children

APOE genotype / Inflammatory marker		3/3	3/4	4/4	P value for comparison of 3/3 and 3/4 genotypes
		n=9	n=9	n=3	
CD68 n=21	GM	364	548	640	0.536
		(349)	(649)	(582)	
	WM	437	363	523	0.659
		(371)	(444)	(539)	
		n=7	n=8	n=3	
CD16 n=18	GM	23	16	61	0.202
		(107)	(18)	(55)	
	WM	24	36	32	0.862
		(98)	(37)	(52)	

Key: Values given are median with mean in brackets, GM – Grey Matter, WM – White Matter, p values – Mann Whitney Test

Table 5.3.4 shows data for CD68 and CD16 quantitation stratified against APOE genotype for HIV **positive** African children. Although there appeared to be APOE ε4 dose related trend for increased CD68 expression in both grey and white matter, these differences did not reach statistical significance when ε3/ε3 and ε3/ε4 cases were compared. For CD16 expression in the grey matter there was little difference

between subjects with ε3/ε3 and ε3/ε4 genotypes. The ε4/ε4 cases were excluded from statistical analysis because of small numbers.

Table 5.3.4 - Correlations between APOE genotype and neuroinflammation in the basal ganglia of HIV positive African children

APOE genotype / Inflammatory marker		ε2/ε3	ε3/ε3	ε3/ε4	ε4/ε4	P value for comparison of 3/3 and 3/4 genotypes
		n=1	n=14	n=5	n=3	
CD68 n=23	GM	1027	355	698	1592	0.105
		(1027)	(478)	(922)	(1607)	
	WM	1100	377	592	1193	0.071
		1100	(363)	(800)	(1146)	
		n=1	n=13	n=5	n=2	
CD16 n=21	GM	50	31	14	21	0.692
		50	(107)	(105)	(21)	
	WM	116	100	450	156	0.844
		116	(284)	(379)	(156)	

Key: Values given are median with mean in brackets, GM – Grey Matter, WM – White Matter, p values – Mann Whitney Test

Fig 5.3.3 CD16 quantitation in basal ganglia in different APOE genotypes – HIV negative (grey boxes) and HIV positive cases (pink boxes)

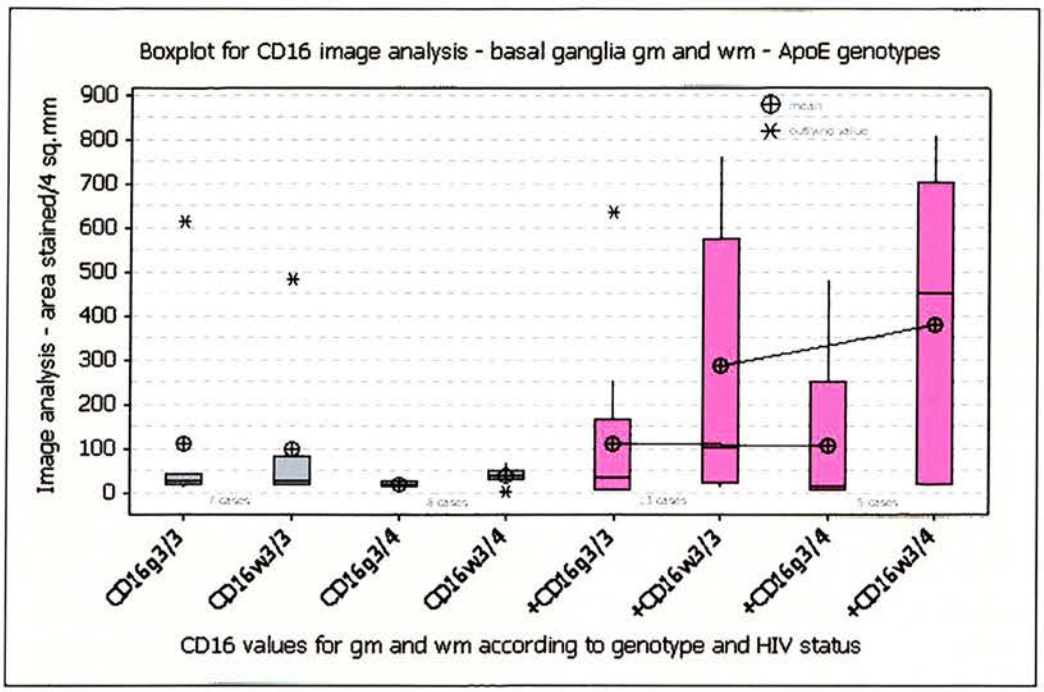


Fig 5.3.3 – this boxplot illustrates the results for CD16 immunostaining of the grey and white matter of the basal ganglia, separated according to APOE genotype and HIV status – the HIV positive boxes are coloured pink. CD staining values are those obtained from image analysis – expressed as the area stained in sq. mu out of a total of 4 sq. mm of section.

The x-axis labels denote grey matter and white matter partition with respect to the genotypes ε3/ε3 and ε3/ε4, so that CD16g3/3 identifies the box expressing the values for the grey matter of those cases found to have an APOE ε3/ε3 genotype. CD16w3/3 identifies the values for the white matter of those with the same genotype – and so on.

Values for cases with an ε2ε3 or ε4ε4 genotype have been excluded due to small numbers. On the graph the mean values have been added. Any outlying values are indicated by an asterisk - *. For the HIV positive cases mean connect line joins the means for the grey matter values of the 3/3 and 3/4 genotypes and the same applies to the means for white matter.

Fig 5.3.4 CD68 quantitation in basal ganglia in different APOE genotypes – HIV negative (grey boxes) and HIV positive cases (pink boxes) image

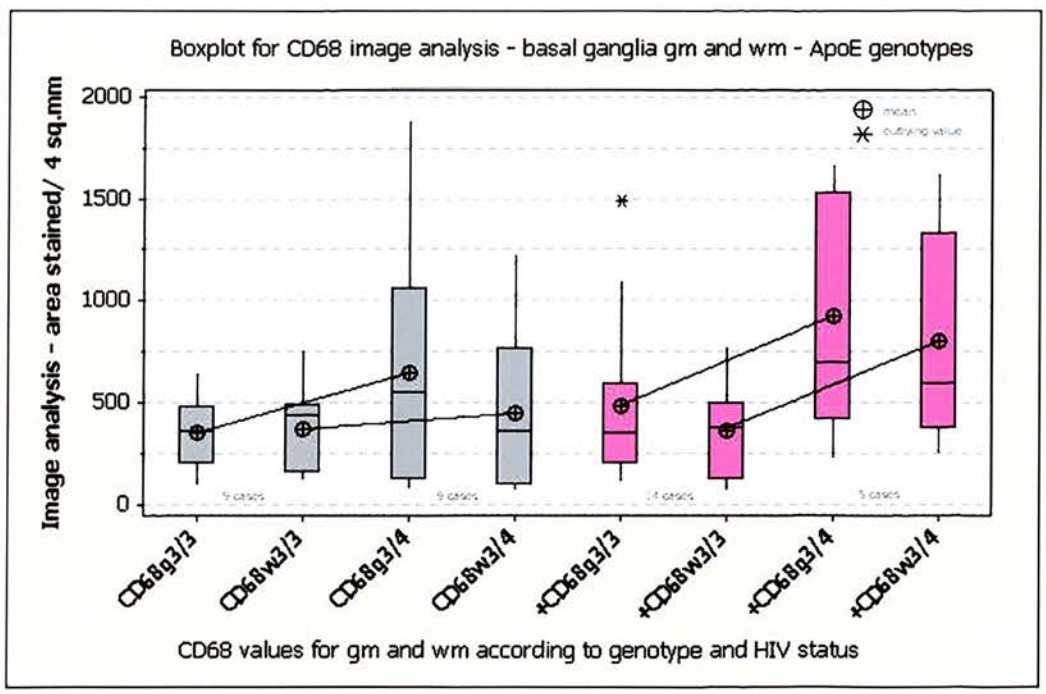


Fig 5.3.4 – this boxplot illustrates the results for CD68 immunostaining of the grey and white matter of the basal ganglia and the same principles of labelling apply as for Fig 5.3.3. A mean connect line links the boxes for grey and white matter separately for HIV negative and positive cases of different APOE genotype.

5.4 - Discussion and Conclusions

These results show that there are statistically significant differences between APOE allele distributions in the African paediatric cohort and in the Scottish population and between the HIV negative and positive children. It is acknowledged that the numbers of African cases genotyped in this study are very small and may not be representative of the population in the Ivory Coast. However the findings in the present study, notably the excess of APOE 4 cases, are in line with what is known of adult West African distributions, where the APOE ε4 frequency may approach 30% (Zekraoui et al. 1997).

For HIV negative children the levels of inflammation, as detected by both CD16 and CD68, differ little between the ε3/ε3, ε3/ε4 and ε4/ε4 genotypes although there is a trend for the CD68 microglial expression to be greater in grey matter in cases with one or more APOE ε4 alleles. For HIV positive children, the corresponding values are generally higher with a similar trend for increase in APOE ε4 cases, albeit in the white matter, but these shifts did not amount to significance. It is worth noting that the APOE ε4/ε4 cases were excluded for statistical analysis because of small numbers. The conclusion is that this study has not been able to demonstrate that the APOE genotype influences the degree of microglial activation in the CNS, either in HIV negative or positive African children. It may be that there is no difference – it should not be assumed that the relationship between microglial activation and APOE genotype demonstrated in AD in Caucasians should necessarily also occur in HIV infection. It may also be that possession of APOE ε4 alleles does not have the same implications in African populations, as exemplified by the study that was unable to

detect a relationship between the APOE ε4 allele and AD in elderly West African males (Osuntokun 1995). Alternatively, there might prove to be a difference but one which could only be shown by examination of a much larger number of subjects.

Among the cases genotyped, HIV negative individuals were fairly balanced against HIV positive children in terms of pathology, both at systemic and CNS levels.

A major difficulty encountered in this study was that of the extraction of DNA from the paraffin wax preserved tissue although this is a recognized method for DNA extraction from archival material (Dunlop et al. 1997). Nevertheless it is well recognised that formalin fixation inhibits the extraction of DNA and subsequent amplification by PCR (Rogers et al. 1990). It is thought that formalin causes cross linkage between DNA and proteins, which may inhibit efficient DNA extraction. Formalin is also known to cause single strand breaks in DNA, which may inhibit efficient DNA replication by PCR (Grafstrom et al. 1983).

The small number of cases is acknowledged as a limitation of the current study, and more extensive sampling of the whole paediatric cohort might provide more robust data. Confirmatory evidence of the association between the APOE ε4 allele and enhanced neuroinflammation might be found in the adult Abidjan autopsy cohort, as well as providing additional information on the APOE allelic distribution in this African population. The difference between the allele frequencies for HIV negative and HIV positive children is noted – the ε4 allele being almost 50% lower in HIV positive children. Even among the 3 cases excluded from the correlations because of unsatisfactory immunohistochemical staining, it is noted that both the HIV negative cases possessed APOE ε4 alleles whereas the excluded HIV positive

case had an APOE ε3/ε3 genotype, in keeping with the apparent greater frequency of ε4 alleles in the former group. It seems unlikely that being HIV positive would influence the dynamics of gene extraction. A bias however could operate at a very early stage – even to affect the vulnerability of an HIV positive foetus, heterozygous or homozygous for the ε4 allele, and thereby the frequency at birth and in the neonatal period. However the numbers are too small to draw any firm conclusions and only a larger survey could clarify this interesting finding.

In concluding this account of the APOE study the results of a few pertinent studies, and their possible applications to neurodegenerative and neurotraumatic human disease, are considered.

Since the effect of APOE on lymphocyte function was investigated in 1980 (Hui et al. 1980) many experimental studies, especially on transgenic mice, have explored the dynamics of the relationship between the APOE polymorphisms and the immune system, particularly in respect of the innate neuroinflammatory response. The absolute importance of APOE was demonstrated in 1999 when it was shown in a study of APOE knockout mice that resistance to systemic infection was impaired. In addition, circulating pro-inflammatory cytokine TNFα levels were increased 4-5 fold in response to bacterial endotoxin lipopolysaccharide (LPS) injection in these mice (de Bont et al. 1999). As an interesting corollary to this finding, a recent paper has described the beneficial effects of ApoE-derived peptides administered to APOE ε2, ε3 and ε4 targeted replacement mice that were subjected to experimental closed head injury (Wang et al. 2007). In these mice, treatment resulted in reduced microglial activation and early inflammatory events, raising the possibility of a therapeutic application of ApoE protein in human cases of head injury.

The physiological basis for this effect was investigated in 2001, when both in-vitro and in-vivo studies showed that ApoE protein down-regulated CNS production of TNFα and Il-1β following the application of LPS (Lynch et al. 2001). The authors thought that this effect of ApoE was specific, and could account for the role that it appears to play in neurological injury and disease.

The effects of differing APOE alleles on the neuronal responses to microglial activation were tested in an experiment using dissociated cultures of glia and neurons from targeted replacement mice bearing the APOE ε2, ε3 and ε4 alleles (Maezawa I – 2006). The degree of neuronal damage was found to be greatest in association with the microglia possessing APOE ε4, and least with the APOE ε2 microglia.

In 2007, a further study on the APOE isoforms and inflammation was published. In a series of experiments, Vitek et al (2007) defined several key features of the APOE gene-modulated macrophage/microglial inflammatory responses using in-vitro and in-vivo models. Microglial cultures from both heterozygous and homozygous targeted replacement mice were studied and it was shown that APOE ε3/ε3 and APOE ε4/ε4 microglia exhibited different morphologies. The former showed a partial ramified shape with extended processes, and the latter the amoeboid morphology shown earlier to be associated with activation (Perry and Gordon 1988).

Vitek et al. confirmed that production of inflammatory mediators was greatest in APOE ε4/ε4 microglial cultures. In addition, they showed that peripheral macrophages shared in this APOE genotype-specific effect. Measurement of supernatant nitrite levels in the cultures revealed a gradation which was shown to be APOE gene-dose dependent – the levels being highest in the APOE ε4/ε4 microglial cultures. They considered the idea that the effect of APOE ε4/ε4 microglia might be,

in part at least, due to reduced production of ApoE protein, thereby depriving the neuropil of its beneficial effects. This concept had previously been proposed in 2001 (Ramassamy et al. 2001). They found, by comparing ApoE protein expression in brain lysates of APOE ε3/ε3 and APOE ε4/ε4 mice that the levels in the former were significantly higher than in the latter. A final finding of interest was that the innate inflammatory response in aged mice tended to be higher in animals with an APOE ε4/ε4 genotype compared with APOE ε3/ε3 animals, as detected by TNFα levels in response to injected LPS in 60-week old mice.

The idea that the innate immune response, as linked to the APOE genotype, and which is considered important for survival in human populations exposed to high levels of pathogens (Finch and Morgan 2007), should become disadvantageous in the elderly is noted (Vitek et al. 2007). However, biological mechanisms that might operate in the elderly at one latitude need not operate at another, where different conditions of climate and diet obtain. Indeed, in West African elderly males, as noted at the beginning of this chapter of this chapter, no association was found between dementia and the APOE ε4 allele (Osuntokun et al. 1995). While interpreting the present data with caution, it may be that links between APOE genotype and microglia observed in Caucasians are not operative in African populations. However the quantitative results for microglial activation do suggest that such a link might be established with a larger study. Finally, when analyzing the distribution of alleles in an autopsy cohort, it should also be considered that the genes being examined might influence survival in this disease-prone population. Accordingly, an autopsy cohort may not necessarily reflect the surviving population.

Conclusions

1. The distribution of APOE alleles in this African cohort, whilst significantly different from that of the Scottish population, and significantly different between HIV positive and negative children, is in keeping with what is known of West African distributions.

2. The results of this study of the relationship between APOE allelic variants and the neuroinflammatory response show a trend for increased activation in APOE ε4 HIV positive cases that does not however reach significance. To our knowledge this is the first study of this relationship in African subjects.

Chapter 6: General Discussion and Conclusions

Introduction

When, in 1991, the authors of the paper " Disease in children infected with HIV in Abidjan, Côte d'Ivoire" (Lucas et al. 1996) started their work they must have been aware of the daunting task that they were about to undertake. There could have been no comparison with contemporary mortuary pathology practice in Europe, either in terms of the harrowing human material and its unremitting volume or the conditions under which they would work, in the absence of air conditioning in a humid tropical city. Nor should the risks of infection, not just from HIV, but also from tuberculosis pass without mention. The documentation of the study alone could scarcely be imagined from the succinct presentation in the paper.

The data gathered at that time remain as a unique archive, both as a resource of HIV material, and also of a spectrum of diseases, both non-tropical and tropical. Many of the non-tropical illnesses are seen in stages of their natural history not described since the pre-antibiotic era in the West. Of course deaths due to meningitis and pneumonia are still recorded in this country but they are few in comparison and most will have had the appropriate treatment. The high frequency of HIV infection noted in the original paper (Lucas et al. 1996) – during the period of the study 80 of the 408 (20%) children brought to the mortuary were HIV positive – underlines the high incidence of HIV infection in infants and children in sub-Saharan Africa. In South Africa alone an estimated 50,000 HIV infected children are born each year (Prendergast et al. 2007).

In 1997, with the publication of the paper " The Neuropathology of HIV-Infected African Children in Abidjan, Côte d'Ivoire' (Bell et al. 1997) the communication of the findings of what was the very first cross-sectional post-mortem survey of HIV in Africa, was completed.

Now, the present study documents three different aspects of the neuropathology. It suffers from several limitations and perhaps the most important is that it was undertaken in the absence of clinical data (there was very little clinical, laboratory or radiological information available to the original authors) or general post mortem findings. Furthermore, no spinal cords were available and thus no conclusions can be drawn as to motor or sensory disability suffered in the months before death. One consistent feature is the remarkably good preservation of the tissue, which was linked to the short death-to-postmortem interval. The decision to study only 40 cases in the Main Study– about a quarter of the whole cohort – has meant smaller numbers for statistical analysis but a reasonably representative sample was obtained by selection on age groups and a detailed survey of inflammatory change has proved possible.

As noted in the introduction to the Main Study there was a differing disease spectrum between the HIV negative and HIV positive groups though a reasonable balance of cases with, and without, significant pathology is thought to obtain. In the original description of the neuropathology of the cohort (Bell et al. 1997), 8 of the 78 HIV positive cases were described as having HIV specific pathology, including 4 cases of HIV encephalitis (HIVE) and 4 cases of leucoencephalopathy. Two of these are subjects in the present study, – one case of HIVE in the Initial Study and another in the Main Study. The abstraction of 5 HIV negative and 5 positive cases which appeared within normal limits neuropathologically, was an attempt to analyse white

matter abnormalities, lymphocytic infiltrates and microglial/macrophage activation in cases free of the confounding factor of disparate disease, thus permitting an assessment of the effects of HIV infection alone on these parameters.

The Initial Study

The usefulness of the initial study may be open to question, not only because of the difficulty in the separation of the glial populations and in reliable counting in the presence of oedema or possible shrinkage, but also because of the natural variation in cell numbers as they appear at different levels in the neuraxis, in different fibre tracts and in different orientations of these fibres. However, the cell counts proved to be reasonably consistent between cases, suggesting that they may reflect an accurate picture of cell subsets in the white matter. Even in the small numbers of cases in this study, a statistically significant difference was detected in cell numbers between the HIV positive and negative cases, the HIV positive counts being the larger. This increase was detected in counts for nuclei posited on their shape or position or GFAP positivity to belong to a class of oligodendrocytes, astrocytes, microglia/macrophages and endothelial cells.

In particular, the numbers of nuclei of GFAP positive cells were increased in the HIV positive cases compared with those that were negative. These results are in agreement with other studies which show that astrocytic hyperplasia is not just confined to the end stage of HIV infection, but occurs throughout the course of the infection in adults (Gray et al. 1996) (Weis et al. 1993). Astrocytosis was also a frequent finding in childhood AIDS encephalopathy (Sharer et al. 1986). One cause of proliferation of astrocytes was investigated in an in-vitro study in 1990 (Selmaj et

al. 1990), which found that serum free TNF-α produced a mitotic response in cultured astrocytes. There was no evidence of cytotoxicity or of proliferation of oligodendrocytes, suggesting that the effect was cell type specific. However, the Main Study showed no evidence of upregulation of GFAP expression in HIV positive cases and this apparent discrepancy is discussed below.

The observed differences in total cell numbers between HIV positive and negative cases reflect increases in all cell types. The reasons for an increase in apparent oligodendrocytes in HIV positive cases are unclear, particularly in the absence of white matter damage as shown in the Main Study, and would require further examination to confirm the finding. However the lack of recognised cell markers for accurate identification of oligodendrocytes remains a stumbling block.

The increase in microglial numbers in HIV positive cases is unsurprising, is borne out by the results in the Main Study and is in keeping with the results of all other studies of HIV/AIDS. However this is the first time that such a finding has been reported for African cases.

The finding that capillary endothelial cell nuclei appear increased in numbers in HIV positive cases compared those of HIV negative cases was unexpected and may signify a subtle inflammatory response. A hundred years after the first microscopical description of inflammation by the German experimental pathologist, Julius Cohnheim (1839-1884), the molecular basis for the changes he observed was investigated (Pober 1988).The first of such studies examined the responses to TNF-α, IL-1, IL-6 and IFN-γ, produced locally by leucocytes, and these included increased BBB permeability and hypertrophy and proliferation of endothelial cells with associated accumulation of intracellular organelles (Stolpen et al. 1986),(Cavender et

al. 1989). In 2001, HIV gp120 was shown to be an activator of brain endothelial cells in children, resulting in increased expression of the endothelial adhesion factors, ICAM-1 and VCAM-1 (Stins et al. 2001). A circulating, virally derived molecule, such as HIV gp120, could induce a proliferative response in endothelial cells and thus account for the apparent increase in numbers of endothelial cells detected in this study. However there are other possible reasons for this result. Firstly, the orientation of the myelin might favour more capillaries being displayed in longitudinal section and thus increasing the numbers of nuclei available to be counted. This seems to be an unlikely explanation since the selection of the areas sampled applied equally to HIV negative and positive cases. Secondly, the endothelial cell nuclei in HIV cases, exposed to molecules such as gp120, might in their activation have become hypertrophied and therefore more visible. Finally, relatively more small vessels may have been affected by vasodilatation in HIV positive cases, rendering their lining endothelial cells more obvious.

The Main Study

This in-depth survey of the inflammatory changes in the brains of African children reveals a wide variation of responses to the infectious diseases that were responsible for their deaths. In most cases, the terminal illnesses are likely to have been of just a few days duration and so the reactions of the adaptive immune system, as judged here by the CD8 and CD20 lymphocyte counts, may have been evolving in many of the subjects. These reactions are subject to many influences, including age, the state of nutrition and hydration, and the co-existence of other disease(s). The polyclonal B-cell activation known to occur during the course of HIV infection is supported in

this study by the finding of B-cells in most of the cases showing low-grade lymphocytic infiltrate and in one of the AIDS cases. Their numbers do not necessarily signify effective function and the impaired humoral immunity associated with HIV infection leads to reduced resistance to bacteria such as H.influezae and S. pneumoniae, which may have caused the deaths of some of these children.

The striking numbers of CD8 lymphocytes in the parenchyma of the brains of the AIDS cases and, of course, those defined by the presence of low-grade lymphocytic infiltrate, suggests that this may represent a response to the presence of HIV infected cells undetected by immunocytochemical methods, as described in a recent paper (McCrossan et al. 2006). By the same token, the numbers seen in the parenchyma in other HIV positive cases, such as those in the category with no significant findings on routine staining, may have a similar explanation.

Investigation of the white matter in the main study did not reveal significant myelin damage, in either the basal ganglia or hippocampus. Similarly there was no evidence of a significant upregulation of GFAP expression in HIV positive cases compared with negative. The results of the Initial Study (albeit in a different group of cases) suggested that significant GFAP positive astrogliosis would be detected in the HIV positive cases in the Main Study and this proved not to be the case. The reasons for the relative lack of GFAP response may be that some of the subjects in the Main Study were less mature than those in the Initial Study and therefore perhaps less capable of mounting an astroglial response. But, in addition, it is noted that cell counting methods reflect cell numbers, whereas GFAP immunostaining indicates upregulated protein expression. Thus the two results may not be as incompatible as

they appear at first sight. In general, it seems from these results that white matter damage is not a major feature of HIVAIDS in untreated African children.

The microglial population of the brain is generally considered to be the local arm of the innate immune system and there is little doubt that evolution has ensured that not only is the territory fully covered by a elaborate spatial gridwork of cells, but also that there is very fine functional tuning (Nimmerjahn et al. 2005). The CD68 and CD16 responses detected in this study can be seen as a snapshot of a mixture of both acute and chronic activation and involving both macrophage (perivascular and bloodstream derived) and microglial populations. The magnitude of these responses, irrespective of the cause, for example in the septicaemia cases in the HIV negative group, constitutes a threat to the CNS. Firstly, in HIV infected individuals the passage of infected monocyte/macrophages is facilitated and secondly, the homeostasis of the parenchyma is prejudiced by cytokine production by activated macrophage/microglia (Glass and Wesselingh 2001). From the CD68 and CD16 results, this study confirms that, even in the context of heightened risk of brain disorders seen in these African children regardless of their HIV status, HIV infection is accompanied by significant activation of microglia and macrophages, constituting the innate immune system.

The importance of these processes, in both the short term survival of the individual and in longer term cognitive functioning, is such that any factor that might influence either process could be critical in determining the outcome. The result of the APOE study is clearly relevant in this regard, given the current interest in dementia and the part that APOE might play in its pathogenesis.

The APOE Study

The main limitation of the APOE study is the number of genotyped cases and any conclusions must be tempered with caution, to be re-examined in the contest of a larger study. However the finding of raised numbers of APOE ε4 cases in the groups overall are in keeping with what is known of African populations. Within this study, the finding of **lower** numbers of APOE ε4 alleles in the HIV positive group is intriguing and might suggest some degree of protection from HIV in individuals bearing this allele although the result is just as likely to be a chance finding.

The results of the APOE study show that within both HIV negative and positive groups, higher levels of CD16 and CD68 expression are generally associated with the APOE ε4 genotype (although it is important to note that the results did not achieve significance) and this tends to support the view that possession of an ε4 allele is likely to be associated an increased level of neuroinflammation. More specifically, the experimental finding that the 4/4 genotype is associated with increased levels of TNF-α production (Vitek et al. 2007) is of contemporary interest in respect of Alzheimer's disease. For several years monoclonal antibodies against inflammatory cytokines, in particular anti-TNF-α, have been used in the treatment of diseases that have a major inflammatory component, such as rheumatoid arthritis. This approach, termed cytokine modulation, is reserved for severe forms of the disease and a drug called Etanercept is one of the more commonly prescribed preparations (BNF 2007). In 2007, a paper was published in which a remarkable improvement in cognitive functioning was reported in patients suffering from Alzheimer's disease following the injection of Etanercept into the peri-spinal venous system (Tobinick 2007). This potentially revolutionary discovery awaits further evaluation in controlled trials. If

substantiated, this finding supports the hypothesis that there is a "pre-structural" and potentially reversible stage in the development of Alzheimer's disease and that this may operate at synaptic level. A similar approach may prove of value in HIV associated dementia (HAD), since a study in 1993 showed that the levels of TNF-α mRNA in the white matter of AIDS patients are significantly greater in HAD than in AIDS patients without dementia, or in controls (Glass et al. 1993).

The novel finding that the degree of neuroinflammation in this African paediatric cohort may have a relationship to the ApoE ε4 allele is of great interest since genetic influences can operate at many levels in determining susceptibility or resistance to disease. Recent views on the location and structure of the blood brain barrier (BBB) suggest that the favoured, if not exclusive, point of transit for inflammatory cells entering the CNS is through the post-capillary venules (Bechmann 2006), and that this is seen as the first step in entry. This passage is apparently made with ease by monocytes in the course of their normal patrolling behaviour. The second step, which involves crossing the perivascular space and the glia limitans, is subject to strict structural and functional constraints. The dynamics of this passage have been explored using a mouse model, and the finding was that mice deficient in CD95 (CD95L is a death ligand expressed constitutively on astrocyte end feet) showed much lower numbers of apoptotic T-cells in experimental allergic encephalitis (EAE) than did control mice (Sabelko et al. 1997). Perivascular apoptosis is a well recognized feature of neuroinflammation (Pender et al. 1991). ApoE is also expressed on the surface of astrocyte end feet (Boyles et al. 1985) (Fujita et al. 1999) and could therefore affect events at the BBB. If immune cell access to the CNS were influenced by ApoE that in turn could affect the level of cytokine

production and the associated tissue disturbance in both Alzheimer's disease and HAD.

Possible Future Extensions of this Study

The results provided by this study are derived from a small part of the original cohort, and it would be of value in the future to document more of the neuroinflammation in this unique archive. Another useful investigation would be to compare the systemic findings and causes of death, described in 1996, with the findings of this study and to extend the APOE survey to the African adults who died in Abidjan. It would also be of great interest to investigate the BBB with immunostaining for evidence of breakdown in HIV positive cases in this cohort, using a marker of tight junctions (ZO 1), and with anti-fibrinogen immunostaining to detect serum leakage.

The APOE study would require larger numbers to provide more definite evidence of a relationship between the ε4 allele and the severity of neuroinflammation in HIV/AIDS in these children.

Epilogue

The material on which this study is based underlies the sad story of HIV/AIDS, especially in children in Africa, but in adults as well and throughout the whole developing world. Even apart from HIV infection it is salutary to reflect on the fact that many of the infants and children in this cohort died of diseases, which, in the West, respond readily to the appropriate treatment.

However, the latest information (UNAIDS – 2007) is that in some parts of Africa, including the Côte d'Ivoire, the prevalence of HIV infection has shown a decline over the last few years. In 2007 there were an estimated 1.7 million (1.4 - 2.4) new infections in sub-Saharan Africa – a significant reduction since 2001. An estimated 22.5 million (20.9 – 24.3) people are living with HIV in Africa and this accounts for 68% of the global total. Elsewhere the figures are not encouraging. In Eastern Europe and Central Asia the number of people living with HIV has increased by 150% in 2007 to 1.6 million (1.2 – 2.1 million). In Asia the fastest growing epidemic is Indonesia. Overall, however the global prevalence has levelled, and the number of new infections has fallen. HIV prevention programmes are considered to have contributed to this improvement. For 2007 the global total of people estimated to be living with HIV was 33.2 million, 2.5 million were thought to have become newly infected and 2.1 million to have died.

Universal access to diagnosis and prevention of mother–to-child transmission are seen as the most important interventions to reduce the number of infected children (Prendergast et al. 2007). Antiretroviral therapy programmes, where they have been available in Africa have usually been successful. In 2004, 544 children in Côte d'Ivoire were started on HAART and a year later 84% were still attending for follow-up (Wemin 2006).

In 2003 it was estimated that 10 million children were dying each year worldwide (Black et al. 2003) of malnutrition and infectious disease. Without public health programmes and political commitment that number is unlikely to fall. The hope is that the goodwill and offers of financial help for the economic development of

countries such as Africa, expressed at the G8 summit in Gleneagles in 2005, will ensure a better future for the world's children.

Conclusions

1. In a small group of this cohort of African children, studied at autopsy, HIV infection was shown to be associated with generalised hyperplasia of both glia and endothelial cells in the white matter
2. A significant increase in the degree and extent of neuroinflammation has been demonstrated in HIV positive children especially in respect of the basal ganglia region and despite a high level of co-present pathology in the brains of both HIV negative and positive children.
3. No white matter abnormality was detected in these developing brains as a result of HIV infection.
4. The distribution of APOE alleles in this African cohort, whilst significantly different from that of the Scottish population, and significantly different between HIV positive and negative children, is in keeping with what is known of West African distributions
5. A trend towards increased activation of the inflammatory response was detected in APOE ε4 HIV positive children.

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APPENDICES: 1-17

1. Monoclonal ABC method
2. Polyclonal ABC method
3. TSA method
4. Immunohistochemistry notes
5. Immunohistochemistry reagents
6. Immunohistochemistry solutions
7. Initial Study – comparison of sampling methods
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14. Main Study – Table of βAPP grades for hippocampus
15. Main Study – Table of GFAP grades for basal ganglia
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17. Main Study – Table of summary statistics for all CD studies

Appendix 1 Monoclonal ABC Method

1. De-wax sections in xylene – two 5 minute immersions.
2. Immerse in 99% ethanol (74 op) – twice for 5 minutes each
3. Immerse in 70% alcohol – twice for 5 minutes each
4. Immerse in picric acid for 20 minutes
5. Wash in running tap water until all the picric acid is removed
6. Immerse in hydrogen peroxide for 10 minutes (50 mls hydrogen peroxide and 450 mls distilled water for 20 minutes (100 mls methanol + 25 mls 3% H O) (blocks endogenous peroxidase enzyme)
7. Wash in water
8. Perform antigen retrieval if required (unmasks antigens which may have been affected by fixation)
9. Wash in water
10. Place sections in sequenza rack with TBS
11. Wash in x1 TBS buffer for 5 minutes
12. Treat with normal rabbit serum (NRS) (1 ml NRS to 4 mls TBS 10 minutes
13. Incubate with primary antibody diluted in NRS for 30 minutes (omit from negative control)
14. Wash in buffer 2 x 5 minutes
15. Incubate in biotinylated rabbit anti-mouse immunoglobulins diluted in NRS for 30 minutes
16. Prepare ABC according to Manufacturer's instruction, 30 minutes prior to use
17. Wash in buffer 2 x 5 minutes

18. Incubate in ABC for 30 mins
19. Wash in buffer 2 x 5 minutes
20. Treat with DAB for 1 – 2 minutes
21. Counterstain in Haematoxylin
22. Dehydrate, clear and mount

Appendix 2 Polyclonal ABC Method

23. De-wax sections in xylene – two 5 minute immersions.
24. Immerse in 99% ethanol (74 op) – twice for 5 minutes each
25. Immerse in 70% alcohol – twice for 5 minutes each
26. Immerse in picric acid for 20 minutes
27. Wash in running tap water until all the picric acid is removed
28. Immerse in hydrogen peroxide for 10 minutes (50 mls hydrogen peroxide and 450 mls distilled water for 20 minutes (100 mls methanol + 25 mls 3% H O) (blocks endogenous peroxidase enzyme)
29. Wash in water
30. Perform antigen retrieval if required (unmasks antigens which may have been affected by fixation)
31. Wash in water
32. Place sections in sequenza rack with TBS
33. Wash in x1 TBS buffer for 5 minutes
34. Treat with normal swine serum (NSS) (1 ml NSS to 4 mls TBS 10 minutes)
35. Incubate with primary antibody diluted in NSS for 30 minutes (omit from negative control)
36. Wash in buffer 2 x 5 minutes
37. Incubate in biotinylated swine anti-rabbit immunoglobulins diluted in NSS for 30 minutes
38. Prepare ABC according to Manufacturer's instruction, 30 minutes prior to use
39. Wash in buffer 2 x 5 minutes

40. Incubate in ABC for 30 mins

41. Wash in buffer 2 x 5 minutes

42. Treat with DAB for 1 – 2 minutes

43. Counterstain in Haematoxylin

44. Dehydrate, clear and mount

Appendix 3 Tyramide signal amplification (TSA) method

45. De-wax sections in xylene – two 5 minute immersions.
46. Immerse in 99% ethanol (74 op) – two 5 minute immersions
47. Immerse in 70% alcohol – two 5 minute immersions
48. Immerse in picric acid for 20 minutes
49. Wash in running tap water until all the picric Acid is removed
50. Immerse in hydrogen peroxide for 10 minutes (50 mls hydrogen peroxide and 450 mls distilled water for 20 minutes (100 mls methanol + 25 mls 3% H O) (blocks endogenous peroxidase enzyme)
51. Wash in water
52. Perform antigen retrieval if required (unmasks antigens which may have been affected by fixation)
53. Wash in water
54. Place sections in sequenza rack with TBS
55. Wash in x1 TBS buffer for 5 minutes
56. Treat with normal rabbit serum (NRS) (1 ml NRS to 4 mls TBS 10 minutes
57. Incubate with TNB for 10 mins
58. Incubate with primary antibody diluted in NRS for 30 minutes (omit from negative control)
59. Rinse in TBS 2 x 5 minutes
60. Incubate in biotinylated rabbit anti-mouse immunoglobulins diluted in NRS for 30 minutes
61. Rinse with TBS 2 x 5 mins
62. Incubate with streptavidin HRP for 30 mins (dilute 1 in 500 in TNB)

63. Rinse with TNT 2 x 5 mins

64. Incubate with biotinylated tyramide for 10 mins

(1μL biotinylated tyramide in 300μL boric acid solution. Boric acid solution is 3mls boric acid + 1μl hydrogen peroxide)

65. Rinse with TNT 2x 5 mins

66. Incubate with streptavidin HRP for 30 mins

67. Rinse with TBS 2 x 5 mins

68. Wash with water

69. Incubate with DAB for 1 – 2 mins

70. Wash with water

71. Counterstain with haematoxylin

72. Dehydrate clear and mount

Appendix 4 Immunohistochemistry notes

1. Slides racks should be agitated once or twice when in xylene, alcohols and picric acid
2. Picric acid breaks formalin bridges – see ApoE chapter
3. Antigen retrieval methods are given in immunostain tables in text. Their aim is to unmask antigen epitopes hidden by formalin fixation
4. TBS is TRIS-HCl buffered saline Ph 7.6 - TRIS (hydroxylmethyl) aminomethane 0.6 gm, sodium chloride 8.1 gm, 1 M HCl 3.8mls and distilled water to 1,000 mls – see reagent sheet
5. The biotinylated secondary antibody attaches the primary antibody to the peroxidase labelled streptavidin-biotin compound ABC.
6. ABC is Avidin Biotin Complex
7. DAB is diaminobenzidine – a chromogenic agent which labels immunoreactive sites a brown colour when oxidised in the presence of streptavidin bound horse radish peroxidase
8. The tyramide signal amplification (TSA) method is used when there are low levels of antigen in the tissue. The principle is that the primary antibody signal can be amplified by the interaction of tyramide with horseradish peroxidase (HRP), which generates large numbers of HRP molecules, This in turn enhances the amount of colour on the addition of DAB thereby increasing the ability to detect smaller amounts of the antigen.
9. TNB and TNT are buffers – see reagent sheet

Appendix 5 Immunohistochemistry Reagents

Product	Company
1kbbp ladder	Invitrogen
ABC peroxidase	Dako
Biotinamidocaproic acid 3-sulpho-N-Hydroxysuccinimide Ester	Sigma
Boric acid	Sigma
Citric acid	Fisher
Cresyl violet	Raymond Lamb
DAB	Vector
DNeasy (DNA extraction kit)	Qiagen
EDTA	Sigma
Ethanol	Fisher
Ethidium bromide	Gibco BRL
Haematoxylin	Surgipath
Hydrogen peroxide	Fisher
Luxol Fast Blue	Raymond Lamb
Agarose	Fisher
Oligonucleotides	Invitrogen
Rabbit serum	Dako
Sodium chloride	Fisher
Tris	Fisher
Tween 20	Sigma

Tyramine Hydrochloride	Sigma
Utrapure water	Sigma
Xylene	Genta

Appendix 6 Solutions for immunohistochemistry and PCR

Solutions

Immunohistochemistry:

Tris buffered saline (TBS) x1		
	Tris-HCL, PH7.6	0.05M
	NaCl	0.15M
	Made up in dH_20	
TNT	Tris HCL pH7.5	0.1M
	NaCl	0.15M
	0.05% Tween 20	
TNB	Tris HCL pH7.5	0.1M
	NaCl	0.15M
	0.5% blocking reagent	
Biotinylated tyramide	4ml Boric acid	50mM
	10mg Biotinamidocaproic acid 3-sulpho-N-Hydroxysuccinimide ester	
	3mg tyramine Hydrochloride	
Hydrogen peroxide		10% v/v
	Made up in dH_20	
Ethanol		99%
		75%
	Made up in dH_20	

PCR:

Loading buffer	Glycerol	49.9% v/v
	10xTBE	49.9% v/v
	Bromophenol blue (BPB)	0.2% w/v
Ethidium bromide (Et Br)		1% w/v
	Made up in dH_20	
Tris-Borate-EDTA (TBE) x10		
	Tris	10.8% w/v
	Boric acid	5.5% w/v
	0.5M EDTA, pH 8.0	4% v/v
	Made up in dH_20	
1kb ladder		200µg/ml
(in 10mM Tris-HCL, pH7.5, 50mM NaCl, 0		

Appendix 7 Initial study – the sampling methods compared

Initial study - the sampling methods compared.		
	First method	Second method
1	derivation – total to cover 1 sq.mm	total to cover $1/5^{th}$ of 1sq. mm
2	frames in x =2	frames in x =1
3	frames in y =2	frames in y = 1
4	width = 328.37 μ	width = 164.18μ
5	height = 246.59μ	height = 123.29μ
6	area = 80975.24 sq.μ	area = 20243.8sq.μ
7	image tiled – in 4 frames	single – untiled image
8	sample – 12.35 fields	sample – 10 fields
9	equation -	equation -
	12.35 images =1sq.mm.	10 images = 202438 sq.μ
		∴for area 1 sq.mm multiply by 4.94
	Total area actually scanned =1sq.mm	Estimation of area scanned = 1sq.mm

Appendix 8

Table of first and second counts of different nuclear subsets with means and SDs

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP
1	**Initial study - the results of the first and second counts with their means.**																																										
2	count	CNS	**c85**				**c61**				**c16**				**c48**				**c31**				**c79**				**c80**				**c40**				**c146**				**c84**			
3	category	region		neg				neg				pos				neg				pos				neg				pos				neg				pos				neg			
4				f 4				f 5				f 5				m 6				m 6				m 7				m 7				m 8				m 8				m 9		
5			1st.	2nd.			1st.	2nd.			1st.	2nd.			1st.	2nd.			1st.	2nd.			1st.	2nd.			1st.	2nd.			1st.	2nd.			1st.	2nd.			1st.	2nd.		
6			total	total	mean	SD	total	total	mean	SD	total	total	mean	SD	total	total	mean	SD	total	total	means	SD	total	total	means	SD	total	total	means	SD	total	total	mean	SD	total	total	means	SD	total	total	means	SD
7																																											
8	total	Cx. gyral core	1103	840	972	186	1051	1044	1048	5	1624	1591	1608	23	1025	*	1025	0	1305	1465	1385	113	1018	825	922	136	1434	1175	1305	183	700	831	766	93	942	942	942	0	962	1003	983	29
9	nuclear	Cx. U-fibres	1249	1195	1222	38	976	860	918	82	1610	1527	1569	59	1099	*	1099	0	1407	1323	1365	59	874	1052	963	126	1078	1058	1068	14	845	691	768	109	1163	1063	1113	71	892	954	923	44
10	counts	hippocampus	1036	983	1010	37	820	904	862	59	1176	1319	1248	101	1161	1018	1090	101	1243	947	1095	209	863	825	844	27	843	1067	955	158	831	702	767	91	1197	1452	1325	180	1042	780	911	185
11		int. cap.	1220	1195	1208	18	814	707	761	76	1361	1275	1318	61	822	801	812	15	919	844	882	53	870	845	858	18	978	1042	1010	45	755	**	755	0	1034	1022	1028	8	1171	988	1080	129
12		cerebellum	1007	949	978	41	861	805	833	40	1048	967	1008	57	960	860	910	71	948	1062	1005	81	609	622	616	9	1349	1156	1253	136	821	763	792	41	797	765	781	23	753	721	737	23
13																																											
14	totals	Cx. gyral core	1073	776	905	210	1051	1008	1030	30	1566	1566	1566	0	982	*	982	0	1272	1432	1352	113	979	785	882	137	1406	1121	1264	202	687	787	737	71	918	894	906	17	936	983	960	33
15	minus	Cx. U-fibres	1206	1170	1188	25	976	850	913	89	1558	1502	1530	40	1060	*	1060	0	1345	1299	1322	33	851	1037	944	132	1053	1023	1038	21	809	686	748	87	1127	1028	1078	70	861	934	898	52
16	endo-	hippocampus	992	968	980	17	820	869	845	35	1110	1279	1195	120	1131	993	1062	98	1184	928	1056	181	838	795	817	30	817	1057	937	170	799	682	741	83	1157	1422	1290	187	1011	771	891	170
17	thelial	int. cap.	1174	1160	1167	10	814	672	743	100	1316	1235	1276	57	806	781	794	18	883	800	842	59	847	805	826	30	947	1032	990	60	716	**	716	0	987	992	990	4	1137	958	1048	127
18	cells	cerebellum	946	909	928	26	861	756	809	74	990	913	953	54	905	830	868	53	912	1032	972	85	583	602	593	13	1300	1126	1214	123	794	743	769	36	743	716	730	19	713	686	700	19
19																																											
20	GFAP	Cx. gyral core	102	79.4	91	16	70	20	45	35	122	84	103	27	85	*	85	0	110	64	87	33	58	74	66	11	95	54	75	29	45	25	35	14	80	64	72	11	63	40	52	16
21	positive	Cx. U-fibres	111	108	110	2	64	59	63	4	137	124	131	9	60	*	60	0	98	64	81	24	61	79	70	13	52	40	46	8	39	64	52	18	76	54	65	16	62	59	61	2
22	cells	hippocampus	89	74	82	11	80	54	67	18	116	99	108	12	51	54	53	2	90	69	81	15	68	25	47	30	51	54	53	2	44	40	42	3	87	64	76	16	48	54	51	4
23		int. cap.	65	89	77	17	35	40	38	4	90	109	100	13	32	54	43	16	64	59	62	4	57	30	44	19	49	49	49	0	47	**	47	0	58	59	59	1	45	40	43	4
24		cerebellum	110	109	110	1	52	99	76	33	143	64	104	56	81	64	73	12	112	128	120	11	78	59	69	13	104	109	107	4	99	54	77	32	90	40	65	35	47	64	56	12
25																																											
26	endo-	Cx. gyral core	30	64	47	24	30	36	33	4	58	25	42	23	43	*	43	0	33	33	33	0	39	40	40	1	28	54	41	18	13	44	29	22	24	49	37	18	26	20	23	4
27	thelial	Cx. U-fibres	43	25	34	13	36	10	23	18	52	25	39	19	39	*	39	0	62	24	43	27	23	15	19	6	25	35	30	7	36	5	21	22	36	35	36	1	31	20	26	8
28	cells	hippocampus	46	15	32	22	28	35	32	5	66	40	53	18	30	25	28	4	59	20	40	28	25	30	28	4	26	10	18	11	32	20	26	8	40	30	35	7	31	9	20	16
29		int. cap.	46	35	41	8	25	35	30	7	45	40	43	4	16	20	18	3	36	44	40	6	23	40	32	12	31	10	22	15	39	**	39	0	47	30	39	12	34	30	32	3
30		cerebellum	61	40	51	15	33	49	41	11	58	54	56	3	55	30	43	18	36	30	33	4	26	20	23	4	49	15	32	24	27	20	24	5	52	49	51	2	40	35	38	4
31																																											
32	round	Cx. gyral core	898	543	721	251	955	978	967	16	1316	1329	1323	9	835	*	835	0	1090	1294	1192	144	904	672	788	164	1288	1027	1158	185	637	736	687	70	779	771	775	6	848	924	886	54
33	/oval	Cx. U-fibres	1023	963	993	42	894	776	835	83	1293	1240	1267	37	915	*	915	0	1178	1146	1162	23	750	934	842	130	976	934	955	30	763	613	688	106	989	919	954	49	782	845	813	45
34	nuclei	hippocampus	828	771	800	40	715	761	738	33	890	1008	949	83	1023	884	954	98	1028	805	917	158	744	746	745	1	734	948	841	151	722	603	663	84	980	1250	1115	191	913	661	787	178
35		int. cap.	1051	1003	1027	34	757	622	690	95	1173	1057	1115	82	760	681	721	56	752	701	727	36	764	766	765	1	885	958	922	52	656	**	656	0	878	889	884	8	1033	860	947	122
36		cerebellum	738	692	715	33	774	618	696	110	752	751	752	1	764	731	748	23	743	805	774	44	464	494	479	21	1166	973	1070	136	670	667	669	2	553	583	568	21	611	582	597	21
37																																											
38	rect-	Cx. gyral core	73	89	81	11	26	10	18	11	128	153	141	8	62	*	62	0	72	74	73	1	17	40	29	16	23	40	32	12	5	30	18	18	60	59	60	1	25	20	23	4
39	angular	Cx. U-fibres	72	99	86	19	18	15	17	2	128	138	133	7	85	*	85	0	69	89	79	14	40	25	33	11	25	15	20	7	7	10	9	2	62	54	58	6	17	30	24	9
40	nuclei	hippocampus	75	123	99	34	25	54	40	21	104	173	139	49	57	54	56	2	66	54	60	8	26	25	26	1	32	54	43	16	33	40	37	5	90	109	100	13	50	54	52	3
41		int. cap.	58	69	64	8	22	10	16	8	53	69	61	11	14	44	29	21	67	40	54	19	26	10	18	11	13	25	19	8	13	**	13	0	51	44	58	5	59	59	59	0
42		cerebellum	98	109	104	8	35	40	38	4	95	99	97	3	60	35	48	18	57	99	78	30	44	49	47	4	30	44	37	10	25	20	23	4	100	94	97	4	55	40	58	11
43																																											

Appendix 9a Table of mean counts, SDs and p values for cell subsets 1, 2 and 3

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	**Initial study - the final results - means, standard deviations and paired t-tests.**																
2	S.	Case code No.	**c85**	**c61**	**c16**	**c48**	**c31**	**c79**	**c80**	**c40**	**c146**	**c84**		HIV -ve	SD	HIV +ve	SD
3														means		means	
4		Age	4	5.5	5.5	6		7.5	7.5	8.5	8.5	9		c61		c16	
5		Sex	f	f	f	m	m	m	m	m	m	m		to		to	
6		HIV	-	-	+	-	+	-	+	-	+	-		c40		c146	
7																	
8	1	total															
9		nuclear counts															
10		Cx. gyral core	952	1063	1608	1025	1385	922	1305	766	943	983		944	133	1310	276
11		Cx. U-fibres	1208	936	1569	1099	1365	963	1068	769	1114	924		942	135	1279	233
12		hippocampus	1012	877	1248	1090	1096	845	955	768	1325	911		895	138	1156	164
13		int. cap.	1208	773	1319	812	882	858	1012	755	1040	1080		800	46	1063	184
14		cerebellum	979	850	1009	912	1005	616	1163	793	781	738		793	127	990	157
15																	
16		means	1072	900	1351	988	1147	841	1101	770	1041	927		paired t-test for means above			
17		SD	126	108	246	123	222	134	138	14	202	125		p=0.000			
18																	
19	2	totals minus															
20		endothelial cells															
21		Cx. gyral core	905	1030	1566	982	1352	882	1264	737	906	960		908	130	1272	275
22		Cx. U-fibres	1188	913	1530	1060	1322	944	1038	748	1078	898		916	129	1242	229
23		hippocampus	980	845	1195	1062	1056	817	937	742	1290	891		867	137	1120	155
24		int. cap.	1167	743	1276	794	842	826	990	716	990	1048		770	50	1025	182
25		cerebellum	928	809	953	868	972	593	1131	769	730	700		760	118	947	165
26																	
27		means	1034	868	1304	953	1109	812	1072	742	999	899		paired t-test for means above			
28		SD	134	109	253	119	222	133	129	19	207	128		p=0.000			
29																	
30	3	GFAP +ve cells															
31		Cx. gyral core	91	45	103	85	87	66	75	35	72	52		58	23	84	29
32		Cx. U-fibres	110	63	131	60	81	70	46	52	65	61		61	7	81	36
33		hippocampus	82	67	108	53	81	47	53	42	76	51		52	11	80	23
34		int. cap.	77	38	100	43	62	44	49	47	59	43		43	4	68	22
35		cerebellum	110	76	104	73	120	69	107	77	65	56		74	4	99	24
36																	
37		means	94	58	109	63	86	59	66	51	67	53		paired t-test for means at			
38		SD	15	16	13	17	21	13	26	16	7	7		p=0.000			
39																	
40	Notes:This table is the upper half of Appendix 9 Cx=cortex. Int cap=internal capsule																
41	Counts are in numbers / sq. mm of section						S=nuclear subsets										

Appendix 9b Table of count means, SDs and p values for nuclear subsets 4, 5 and 6

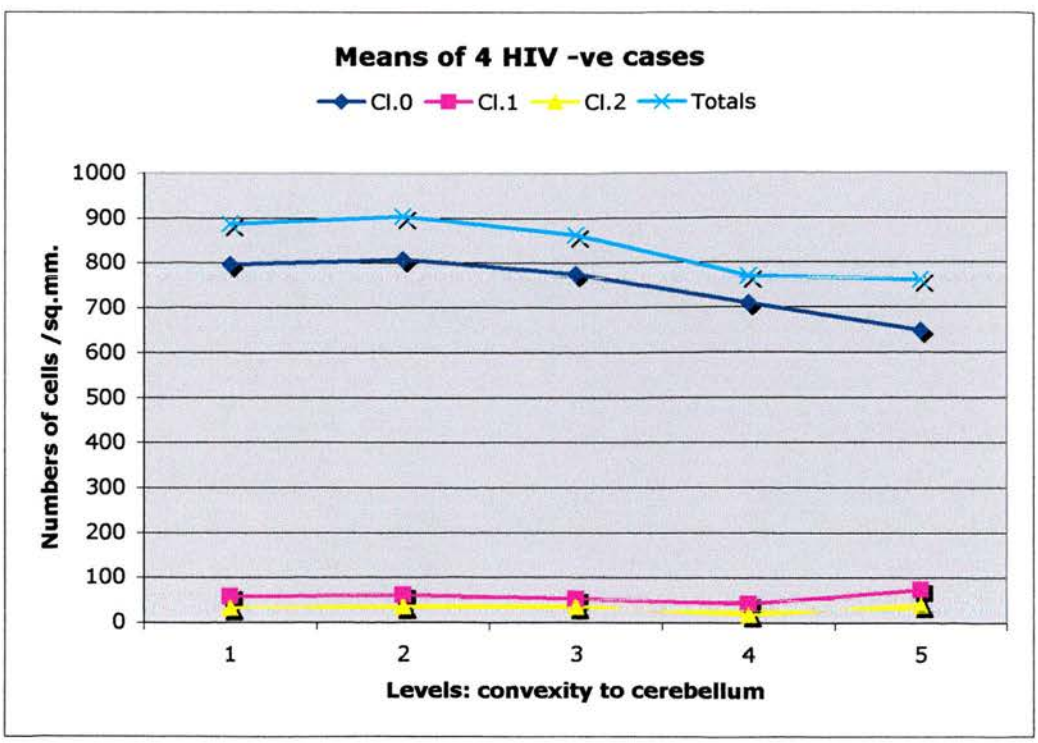
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	**Initial study - the final results - means, standard deviations and paired t-tests.**																
2	S.	Case code No.	**c85**	**c61**	**c16**	**c48**	**c31**	**c79**	**c80**	**c40**	**c146**	**ccn 84**		HIV -ve	SD	HIV +ve	SD
3														means		means	
4		Age	4	5.5	5.5	6	6	7.5	7.5	8.5	8.5	9		c61		c16	
5		Sex	f	f	f	m	m	m	m	m	m	m		to		to	
6		HIV	-	-	+	-	+	-	+	-	+	-		c40		c146	
7																	
8	4	endothelial cells															
9		Cx. gyral core	47	33	42	43	33	40	41	29	37	23		36	6	38	4
10		Cx. U-fibres	34	23	39	39	43	19	30	21	36	26		26	9	37	5
11		hippocampus	32	32	53	28	40	28	18	26	35	20		29	3	37	14
12		int. cap.	41	30	43	18	40	32	22	39	39	32		30	9	36	9
13		cerebellum	51	41	56	43	33	23	32	24	51	38		33	11	43	12
14																	
15		means	41	32	47	34	38	28	29	28	40	28		paired t-test for means above			
16		SD	8	6	7	11	5	8	9	7	7	7		p=0.007			
17																	
18	5	round nuclei															
19		Cx. gyral core	721	967	1323	835	1192	788	1158	687	775	886		819	116	1112	236
20		Cx. U-fibres	993	835	1267	915	1162	842	955	688	954	813		820	95	1085	156
21		hippocampus	800	738	949	954	917	745	841	663	1115	787		775	125	956	116
22		int. cap.	1027	690	1115	721	727	765	922	656	884	947		708	46	912	159
23		cerebellum	715	696	752	748	774	479	1070	669	568	597		648	117	791	208
24																	
25		means	851	785	1081	835	954	724	989	673	859	806		paired t-test for means above			
26		SD	149	117	235	101	215	141	125	14	204	133		p=0.000			
27																	
28	6	rect. Nuclei															
29		Cx. gyral core	81	18	141	62	73	29	32	18	60	23		32	21	77	46
30		Cx. U-fibres	86	17	133	85	79	33	20	9	58	24		36	34	73	47
31		hippocampus	99	40	139	56	60	26	43	37	100	52		40	12	86	43
32		int. cap.	64	16	61	29	54	18	19	13	58	59		19	7	48	20
33		cerebellum	104	38	97	48	78	47	37	23	97	58		39	12	77	28
34																	
35		means	87	26	114	56	69	31	30	20	75	43		paired t-test for means above			
36		SD	16	12	35	20	11	11	11	11	22	18		p=0.000			
37																	
38	Notes:This table is the lower half of Appendix 9 Cx=cortex. Int cap=internal capsule																
39	Counts are in numbers / sq. mm of section						S=nuclear subset										

Initial study - the means of the HIV negative cases compared for CNS areas.

CNS area	mean Cl.0	%	mean Cl. 1	%	mean Cl.2	%		Totals
Cx.gyral core	797	90%	58	6%	32	4%		887
Cx. U fibre z.	807	89%	61	7%	36	4%		904
Temp.lobe	775	90%	52	6%	36	4%		863
Int.Cap.	708	92%	43	6%	19	2%	*	770
Cerebellum	648	85%	74	10%	39	5%	**	761
rounding	800		60		30			
totals	800		60		35			
	775		50		35			
	700		45		20			
	650		75		40			
percentages		90%		6%		4%		
		89%		7%		4%		
		90%		6%		4%		
Int.Cap. **		92%		6%		2%		
Cerebellum *		85%		10%		5%		
mean percentages -approximate		90%		6%		4%		

Notes * internal capsule - oblique fibre orientation - fewer nuclei?
** cerebellum - oedema - nuclear fallout? Reduced numbers?

Cl.0=round nuclei - Cl.1=GFAP positive cells - Cl.2= rectangular nuclei



	A	B	C	D	E	F
1	**Basal ganglia LFB light-box grades**					
2	**Code No**	**Age**	**Sex**	**HIV**	**Grd**	**CNS pathology findings on routine staining**
3						
4	**HIV negative cases**					
5	c54	0.4	M	-	1	meningoencephalitis and infarcts
6	c94	0.5	M	-	1	no significant findings
7	c100	0.5	F	-	1	no significant findings
8	c104	6.0	M	-	1	cerebral malaria
9	c109	0.5	F	-	1	purulent meningitis
10	c113	0.4	M	-	1	no significant findings
11	c59	1.6	M	-	2	purulent meningitis
12	c77	0.3	F	-	2	possible septicaemia
13	c89	0.5	F	-	2	purulent meningoencephalitis
14	c93	1.5	F	-	2	no significant findings
15	c117	2.9	M	-	2	cerebral malaria
16	c33	1.6	F	-	3	possible septicaemia
17	c42	5.0	F	-	3	no significant findings
18	c50	1.6	F	-	3	no significant findings
19	c57	5.0	F	-	3	no significant findings
20	c60	1.5	F	-	3	no significant findings
21	c67	4.0	F	-	3	no significant findings
22	c74	1.5	F	-	3	no significant findings
23	c76	1.6	M	-	3	no significant findings
24	c101	4.0	M	-	3	possible septicaemia
25						
26			Mean=2.15			p=0.666 (Mann-Whitney)
27			SD=0.875			
28						
29	**HIV positive cases**					
30	c5	1.7	F	+	1	no significant findings
31	c82	0.2	F	+	1	cerebellar infarcts & micro-haemorrhages
32	c110	0.4	M	+	1	toxoplasmosis
33	c129	0.4	F	+	1	low grade lymphocytic infiltrate
34	c131	0.4	M	+	1	low grade encephalitis
35	c24	5.0	M	+	2	measles encephalitis
36	c62	4.0	F	+	2	low grade lymphocytic infiltrate
37	c121	1.6	M	+	2	cerebral oedema
38	c122	3.3	F	+	2	low grade lymphocytic infiltrate
39	c125	1.5	M	+	2	no significant findings
40	c126	1.5	F	+	2	no significant findings
41	c140	4.0	M	+	2	no significant findings
42	c150	0.4	F	+	2	no significant findings
43	c157	0.2	M	+	2	purulent meningitis
44	c8	3.5	F	+	3	subtle widespread wm damage
45	c9	5.1	F	+	3	medullitis
46	c55	1.7	M	+	3	low grade lymphocytic infiltrate
47	c91	1.6	M	+	3	low grade lymphocytic infiltrate
48	c128	1.8	F	+	3	low grade lymphocytic infiltrate
49	c152	6.4	M	+	3	HIV encephalitis & toxoplasmosis
50						
51			Mean=2.05			
52			SD=0.76			
53						
54	Note of grading:		1=minimal stain intensity or extent.			
55			2=moderate stain intensity or extent.			
56			3=normal or almost normal stain intensity or extent			

	A	B	C	D	E	F
1	**Hippocampus LFB light-box grades**					
2	**Code No**	**Age**	**Sex**	**HIV**	**Grd**	**CNS Pathology findings on routine staining**
3						
4	**HIV negative cases**					
5	c52	0.5	M	-	1	no significant findings
6	c54	0.4	M	-	1	meningoencephalitis & infarcts
7	c60	1.5	F	-	1	no significant findings
8	c77	0.3	F	-	1	possible septicaemia
9	c94	0.5	M	-	1	no significant findings
10	c100	0.5	F	-	1	no significant findings
11	c109	0.5	F	-	1	purulent meningitis
12	c23	1.5	M	-	2	no significant findings
13	c33	1.6	F	-	2	possible septicaemia
14	c50	1.6	F	-	2	no significant findings
15	c63	2.5	F	-	2	no significant findings
16	c89	0.5	F	-	2	purulent meningoencephalitis
17	c57	5.0	F	-	3	no significant findings
18	c59	1.6	M	-	3	purulent meningitis
19	c86	1.8	F	-	3	no significant findings
20	c93	1.5	F	-	3	no significant findings
21	c101	4.0	M	-	3	possible septicaemia
22	c104	6.0	M	-	3	cerebral malaria
23	c117	2.9	M	-	3	cerebral malaria
24	c124	5.0	M	-	3	no significant findings
25						
26			Mean=2.05			p=0.840 (Mann-Whitney)
27			SD=0.89			
28						
29	**HIV positive cases**					
30	c51	1.8	F	+	1	low grade lymphocytic infiltrate
31	c82	0.2	F	+	1	cerebellar infarct & microhaemorrhages
32	c110	0.4	M	+	1	toxoplasmosis
33	c150	0.4	F	+	1	nothing significant
34	c157	0.2	M	+	1	purulent meningitis
35	c5	1.7	F	+	2	no significant findings
36	c9	5.1	F	+	2	medullitis
37	c16	5.3	F	+	2	no significant findings
38	c55	1.7	M	+	2	low grade lymphocytic infiltrate
39	c62	4.0	F	+	2	low grade lymphocytic infiltrate
40	c70	2.1	F	+	2	low grade lymphocytic infiltrate
41	c91	1.6	M	+	2	low grade lymphocytic infiltrate
42	c126	1.5	F	+	2	no significant findings
43	c129	0.4	F	+	2	low grade lymphocytic infiltrate
44	c131	0.4	M	+	2	low grade encephalitis
45	c8	3.5	F	+	3	subtle widespread wm damage
46	c24	5.0	M	+	3	measles encephalitis
47	c122	3.3	F	+	3	low grade lymphocytic infiltrate
48	c125	1.5	M	+	3	no significant findings
49	c140	4.0	M	+	3	no significant findings
50						
51			Mean=2			
52			SD=0.73			
53						
54	Note of grading:		1=minimal stain intensity or extent.			
55			2=moderate stain intensity or extent.			
56			3=normal or almost normal stain intensity or extent			

	A	B	C	D	E	F
1	**Basal ganglia βAPP grades**					
2	**Code No**	**Age**	**Sex**	**HIV**	**grd**	**CNS pathology findings on routine staining**
3						
4	**HIV negative cases**					
5	c42	5.0	F	-	0	no significant findings
6	c50	1.6	F	-	0	no significant findings
7	c57	5.0	F	-	0	no significant findings
8	c59	1.6	M	-	0	purulent meningitis
9	c67	4.0	F	-	0	no significant findings
10	c74	1.5	F	-	0	no significant findings
11	c76	1.6	M	-	0	no significant findings
12	c77	0.3	F	-	0	possible septicaemia
13	c89	0.5	F	-	0	purulent meningoencephalitis
14	c94	0.5	M	-	0	no significant findings
15	c100	0.5	F	-	0	no significant findings
16	c101	4.0	M	-	0	possible septicaemia
17	c104	6.0	M	-	0	cerebral malaria
18	c113	0.4	M	-	0	no significant findings
19	c117	2.9	M	-	1	cerebral malaria
20	c33	1.6	F	-	2	possible septicaemia
21	c60	1.5	F	-	2	no significant findings
22	c109	0.5	F	-	2	purulent meningitis
23	c54	0.4	M	-	3	meningoencephalitis and infarcts
24	c93	1.5	F	-	3	no significant findings
25						
26		Mean=2.17				**p=0.029 (Mann-Whitney)**
27		SD=0.75				
28						
29	**HIV positive cases**					
30	c5	1.7	F	+	0	no significant findings
31	c8	3.5	F	+	0	subtle widespread wm damage
32	c9	5.1	F	+	0	medullitis
33	c62	4.0	F	+	0	low grade lymphocytic infiltrate
34	c82	0.2	F	+	0	cerebellar infarcts & micro-haemorrhages
35	c91	1.6	M	+	0	low grade lymphocytic infiltrate
36	c110	0.4	M	+	0	toxoplasmosis
37	c121	1.6	M	+	0	cerebral oedema
38	c122	3.3	F	+	0	low grade lymphocytic infiltrate
39	c125	1.5	M	+	0	no significant findings
40	c128	1.8	F	+	0	low grade lymphocytic infiltrate
41	c131	0.4	M	+	0	low grade encephalitis
42	c140	4.0	M	+	0	no significant findings
43	c157	0.2	M	+	0	purulent meningitis
44	c24	5.0	M	+	1	measles encephalitis
45	c55	1.7	M	+	1	low grade lymphocytic infiltrate
46	c126	1.5	F	+	1	no significant findings
47	c129	0.4	F	+	1	low grade lymphocytic infiltrate
48	c150	0.4	F	+	1	no significant findings
49	c152	6.4	M	+	2	HIV encephalitis & toxoplasmosis
50						
51						
52		Mean=1.17				
53		SD=0.41				
54						
55	Notes:grd=overall grade of staining. 1=minimal, 2=moderate and 3=maximal.					

	A	B	C	D	E	F
1	**Hippocampus - βAPP grades**					
2	**Code No**	**Age**	**Sex**	**HIV**	**grd**	**CNS pathology findings on routine staining**
3						
4	**HIV negative cases**					
5	c23	1.5	M	-	0	no significant findings
6	c50	1.6	F	-	0	no significant findings
7	c52	0.5	M	-	0	no significant findings
8	c57	5.0	F	-	0	no significant findings
9	c59	1.6	M	-	0	purulent meningitis
10	c86	1.8	F	-	0	no significant findings
11	c89	0.5	F	-	0	purulent meningoencephalitis
12	c94	0.5	M	-	0	no significant findings
13	c100	0.5	F	-	0	no significant findings
14	c101	4.0	M	-	0	possible septicaemia
15	c104	6.0	M	-	0	cerebral malaria
16	c33	1.6	F	-	1	possible septicaemia
17	c77	0.3	F	-	1	possible septicaemia
18	c109	0.5	F	-	1	purulent meningitis
19	c124	5.0	M	-	1	no significant findings
20	c54	0.4	M	-	2	meningoencephalitis & infarcts
21	c63	2.5	F	-	2	no significant findings
22	c93	1.5	F	-	2	no significant findings
23	c117	2.9	M	-	2	cerebral malaria
24	c60	1.5	F	-	3	no significant findings
25						
26		Mean=1.2				p=0.585 (Mann-Whitney)
27		SD=0.71				
28						
29	**HIV positive cases**					
30	c5	1.7	F	+	0	no significant findings
31	c8	3.5	F	+	0	subtle widespread wm damage
32	c9	5.1	F	+	0	medullitis
33	c16	5.3	F	+	0	no significant findings
34	c51	1.8	F	+	0	low grade lymphocytic infiltrate
35	c82	0.2	F	+	0	cerebellar infarct & microhaemorrhages
36	c122	3.3	F	+	0	low grade lymphocytic infiltrate
37	c125	1.5	M	+	0	no significant findings
38	c131	0.4	M	+	0	low grade encephalitis
39	c150	0.4	F	+	0	nothing significant
40	c24	5.0	M	+	1	measles encephalitis
41	c110	0.4	M	+	1	toxoplasmosis
42	c126	1.5	F	+	1	no significant findings
43	c129	0.4	F	+	1	low grade lymphocytic infiltrate
44	c140	4.0	M	+	1	no significant findings
45	c157	0.2	M	+	1	purulent meningitis
46	c62	4.0	F	+	2	low grade lymphocytic infiltrate
47	c70	2.1	F	+	2	low grade lymphocytic infiltrate
48	c91	1.6	M	+	2	low grade lymphocytic infiltrate
49	c55	1.7	M	+	3	low grade lymphocytic infiltrate
50						
51		Mean=1.5				
52		SD=0.71				
53						
54	Notes:grd=overall grade of staining. 1=minimal, 2=moderate and 3=maximal.					

	A	B	C	D	E	F
1	**Basal ganglia - GFAP grades**					
2	**Code No**	**Age**	**Sex**	**HIV**	**Grade**	**CNS pathology findings on routine staining**
3						
4	**HIV negative cases**					
5	c33	1.6	F	-	1	possible septicaemia
6	c42	5.0	F	-	1	no significant findings
7	c54	0.4	M	-	1	meningoencephalitis and infarcts
8	c67	4.0	F	-	1	no significant findings
9	c74	1.5	F	-	1	no significant findings
10	c76	1.6	M	-	1	no significant findings
11	c77	0.3	F	-	1	possible septicaemia
12	c89	0.5	F	-	1	purulent meningoencephalitis
13	c100	0.5	F	-	1	no significant findings
14	c101	4.0	M	-	1	possible septicaemia
15	c104	6.0	M	-	1	cerebral malaria
16	c50	1.6	F	-	2	no significant findings
17	c57	5.0	F	-	2	no significant findings
18	c59	1.6	M	-	2	purulent meningitis
19	c93	1.5	F	-	2	no significant findings
20	c109	0.5	F	-	2	purulent meningitis
21	c113	0.4	M	-	2	no significant findings
22	c117	2.9	M	-	2	cerebral malaria
23	c60	1.5	F	-	3	no significant findings
24	c94	0.5	M	-	3	no significant findings
25						
26			Mean=1.55			p=0.151 (Mann-Whitney)
27			SD=0.69			
28						
29	**HIV positive cases**					
30	c8	3.5	F	+	1	subtle widespread wm damage
31	c9	5.1	F	+	1	medullitis
32	c55	1.7	M	+	1	low grade lymphocytic infiltrate
33	c91	1.6	M	+	1	low grade lymphocytic infiltrate
34	c125	1.5	M	+	1	no significant findings
35	c140	4.0	M	+	1	no significant findings
36	c150	0.4	F	+	1	no significant findings
37	c62	4.0	F	+	2	low grade lymphocytic infiltrate
38	c110	0.4	M	+	2	toxoplasmosis
39	c122	3.3	F	+	2	low grade lymphocytic infiltrate
40	c126	1.5	F	+	2	no significant findings
41	c128	1.8	F	+	2	low grade lymphocytic infiltrate
42	c129	0.4	F	+	2	low grade lymphocytic infiltrate
43	c131	0.4	M	+	2	low grade encephalitis
44	c152	6.4	M	+	2	HIV encephalitis & toxoplasmosis
45	c5	1.7	F	+	3	no significant findings
46	c24	5.0	M	+	3	measles encephalitis
47	c82	0.2	F	+	3	cerebellar infarcts & micro-haemorrhages
48	c121	1.6	M	+	3	cerebral oedema
49	c157	0.2	M	+	3	purulent meningitis
50						
51			Mean=1.9			
52			SD=0.79			
53	Notes: grade 1=absent to minimal expression.					
54	grade 2=slight expression					
55	grade 3=moderate expression					
56	grade 4=extensive and strong expression					

	A	B	C	D	E	F
1	Hippocampus - GFAP grades					
2	Code No	Age	Sex	HIV	grade	CNS pathology findings on routine staining
3						
4	HIV negative cases					
5	c23	1.5	M	-	1	no significant findings
6	c33	1.6	F	-	1	possible septicaemia
7	c50	1.6	F	-	1	no significant findings
8	c52	0.5	M	-	1	no significant findings
9	c54	0.4	M	-	1	meningoencephalitis & infarcts
10	c89	0.5	F	-	1	purulent meningoencephalitis
11	c94	0.5	M	-	1	no significant findings
12	c63	2.5	F	-	2	no significant findings
13	c86	1.8	F	-	2	no significant findings
14	c101	4	M	-	2	possible septicaemia
15	c104	6	M	-	2	cerebral malaria
16	c124	5	M	-	2	no significant findings
17	c57	5	F	-	3	no significant findings
18	c59	1.6	M	-	3	purulent meningitis
19	c60	1.5	F	-	3	no significant findings
20	c77	0.3	F	-	3	possible septicaemia
21	c93	1.5	F	-	3	no significant findings
22	c100	0.5	F	-	3	no significant findings
23	c117	2.9	M	-	3	cerebral malaria
24	c109	0.5	F	-	4	purulent meningitis
25						
26			Mean=2			p=0.120 (Mann-Whitney)
27			SD=0.95			
28						
29	HIV positive cases					
30	c16	5.3	F	+	1	no significant findings
31	c55	1.7	M	+	1	low grade lymphocytic infiltrate
32	c140	4	M	+	1	no significant findings
33	c5	1.7	F	+	2	no significant findings
34	c24	5	M	+	2	measles encephalitis
35	c51	1.8	F	+	2	low grade lymphocytic infiltrate
36	c70	2.1	F	+	2	low grade lymphocytic infiltrate
37	c82	0.2	F	+	2	cerebellar infarct & microhaemorrhages
38	c110	0.4	M	+	2	toxoplasmosis
39	c122	3.3	F	+	2	low grade lymphocytic infiltrate
40	c8	3.5	F	+	3	subtle widespread wm damage
41	c9	5.1	F	+	3	medullitis
42	c62	4	F	+	3	low grade lymphocytic infiltrate
43	c91	1.6	M	+	3	low grade lymphocytic infiltrate
44	c125	1.5	M	+	4	no significant findings
45	c126	1.5	F	+	4	no significant findings
46	c129	0.4	F	+	4	low grade lymphocytic infiltrate
47	c131	0.4	M	+	4	low grade encephalitis
48	c150	0.4	F	+	4	no significant findings
49	c157	0.2	M	+	4	purulent meningitis
50						
51			Mean=2.65			
52			SD=1.09			
53	Notes: grade 1=absent to minimal expression.					
54	grade 2=slight expression					
55	grade 3=moderate expression					
56	grade 4= extensive and strong expression					

Appendix 17 Table of summary statistics for the Main Study

	A	B	C	D	E	F	G	H	I
1	**Basal ganglia and hippocampus - summary of Mann-Whitney test results**								
2									
3	**CD8 counts**					**CD8 counts**			
4	basal ganglia Mann-Whitney tests					hippocampus Mann-Whitney tests			
5			gm medians	wm medians				gm medians	wm medians
6	Group A1	HIV -ve	5	5		Group A2	HIV -ve	3	9
7	pv cells	HIV +ve	19.5	23		pv cells	HIV +ve	11	32
8		p value	**p=0.001**	**p=0.000**			p value	**p=0.002**	**p=0.000**
9									
10			gm medians	wm medians				gm medians	wm medians
11	Group A1	HIV -ve	0	0		Group A2	HIV -ve	0	0
12	par cells	HIV +ve	1	13		par cells	HIV +ve	2	4
13		p value	**p=0.011**	**p=0.000**			p value	**p=0.003**	**p=0.006**
14									
15			gm medians	wm medians				gm medians	wm medians
16	Group B1	HIV -ve	5	7		Group B2	HIV -ve	3	14
17	pv cells	HIV +ve	17	20		pv cells	HIV +ve	6	24
18		p value	p=0.115	p=0.060			p value	p=0.172	p=0.071
19									
20			gm medians	wm medians				gm medians	wm medians
21	Group B1	HIV -ve	0	0		Group B2	HIV -ve	0	0
22	par cells	HIV +ve	1	10		par cells	HIV +ve	0	21
23		p value	p=0.251	**p=0.034**			p value	p=0.441	**p=0.011**
24									
25									
26	**CD20 counts**					**CD20 counts**			
27	basal ganglia Mann-Whitney tests					hippocampus Mann-Whitney tests			
28			gm medians	wm medians				gm medians	wm medians
29	Group A1	HIV -ve	1.5	0		Group A2	HIV -ve	1.5	2.5
30	pv cells	HIV+ve	2.5	0.5		pv cells	HIV+ve	1	2.5
31			p=0.471	p=0.895				p=0.651	p=0.913
32									
33			gm medians	wm medians				gm medians	wm medians
34	Group B1	HIV -ve	2	0		Group B2	HIV -ve	2	1
35	pv cells	HIV +ve	3	1		pv cells	HIV +ve	0	1
36			p=0.832	p=0.908				0.158	p=0.295
37									
38									
39	**CD14 grades**					**CD14 grades**			
40	basal ganglia Mann-Whitney tests					hippocampus Mann-Whitney tests			
41			medians						
42	Group A1	HIV-ve	2			Group A2	HIV-ve	2.25	
43		HIV+ve	3				HIV+ve	2	
44			p=0.338					p=0.490	
45									
46	Group B1	HIV-ve	2			Group B2	HIV-ve	2	
47		HIV+ve	2				HIV+ve	2	
48			p=0.259					0.443.	
49									
50									
51	**CD16 image analysis**					**CD16 image analysis**			
52	basal ganglia Mann-Whitney tests					hippocampus Mann-Whitney tests			
53			gm medians	wm medians				gm medians	wm medians
54	Group C	HIV -ve	18	32		Group A2	HIV -ve	36	37
55		HIV +ve	28	347.5			HIV +ve	142	208
56		p value	p=0.447	**p=0.03**			p value	**p=0.037**	**p=0.004**
57									
58									
59			gm medians	wm medians				gm medians	wm medians
60	Group B1	HIV -ve	18	26		Group B2	HIV -ve	17	18
61		HIV +ve	15	95			HIV +ve	49	209
62		p value	p=0.753	p=0.210			p value	p=0.295	p=0.346
63									
64									
65	**CD68 image analysis results**					**CD68 image analysis results**			
66	Basal ganglia - Mann-Whitney tests					Hippocampus - Mann-Whitney tests			
67									
68			gm medians	wm medians				gm medians	wm medians
69	Group A1	HIV -ve	380	373		Group A2	HIV -ve	697	458
70		HIV +ve	1063	761			HIV +ve	826	837
71		p value	**p=0.002**	**p=0.009**			p value	p=0.091	p=0.468
72									
73									
74	Group B1	HIV -ve	364	383		Group B2	HIV -ve	157	244
75		HIV +ve	1076	698			HIV +ve	716	810
76		p value	p=0.142	p=0.403			p value	p=0.144	**p=0.022**
77									
78	Notes: for Groups A1 and A2 n=20								
79	for Groups B1 and B2 n=5								
80	for Group C n=17								
81									